University of Alberta

Constitutive expression of *ABR17* cDNA enhances germination and promotes early flowering in *Brassica napus*

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science
in
Plant Science

Department of Agricultural, Food and Nutritional Science

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Edmonton, Alberta
Fall 2007

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ISBN: 978-0-494-33237-5
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ISBN: 978-0-494-33237-5

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Abstract

Pathogenesis–Related (PR) proteins are induced in a number of angiosperms and gymnosperms after exposure to abiotic or biotic stresses, but their biological function remains unknown. In the present study, we report the constitutive expression of *Pisum sativum* ABA-responsive 17 (*ABR17/ PR 10.4*) cDNA in a DH (doubled haploid) *Brassica napus* (canola) and characterized the transgenic line. We observed significantly ($P > 0.05$) greater germination in the transgenic line at 275 mM NaCl, 5 °C, and 10 °C + 75 mM NaCl when compared to the wild type. In addition, we observed a significantly greater rate of flowering, earlier flowering and greater plant height in the transgenic lines when compared to the wild type. Proteome analysis of our transgenic lines does not reveal considerable transgene-induced changes although carbonic anhydrase, which may be responsible for some of the observed characteristics, was identified as being elevated. Our findings are discussed within the context of known biological activities of PR 10 proteins.

Acknowledgements

I would like to express my sincere appreciation to my graduate supervisor Dr. Nat Kav. His encouragement, guidance, enthusiasm, expertise, and financial support were invaluable to me throughout the course of my graduate program. I would also like to thank Dr. Saleh Shah for his support and assistance during my studies, as well as, my committee members Dr. Randall Weselake and Dr. Michael Deyholos.

I would like to express my gratitude for the technical support and friendship provided to me by my fellow graduate students Sanjeeva Srivastava, William Yajima, Mohsen Mohammadi, Sowmya Krishnaswamy, Yue Liang, Nidhi Sharma, and Bo Yang. I would also like to thank Dr. Hafiz Rahman, Dr. Laki Goonewardene, Dr. Steven Strelkov, Dr. Dean Spaner, Melinda Hollis, Govind Pandompatam, Shawna Kelly, and Erin Carroll for their encouragement and support throughout my studies.

Finally, I would like to thank my parents, Dianne and Warren Dunfield, for their tireless support and understanding.

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List of Abbreviations

ABA	Abscisic Acid
ABR17	ABA-responsive proteins 17
ABRE	Abscisic acid responsive element
ANS	8-anilino-1-naphthalenesulfonic acid
ANOVA	Analysis of variance
ANCOVA	Analysis of co-variance
APX	Ascorbate peroxidase
BSA	Bovine serum albumin
CA	Carbonic anhydrase
CBF	C-repeat binding factors
CDPK	Ca^{2+} dependent protein kinases
CK	Cytokinin
COR	Cold regulated
CRT	C-repeat
CSBP	Cytokinin-specific binding proteins
DAP	Days after planting
DEAE	Diethlyaminoethyl
DH	Doubled haploid
DRE	Drought responsive element
DREB	DRE-binding factor
DTT	Dithiothreitol

ESI	Electrospray ionization
EST	Expressed sequence tag
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
GA	Gibberellic acid
GPX	Glutathione peroxidase
GRP	Glycine-rich RNA binding protein
GST	Glutathione S-transferase
HSP	Heat shock proteins
LEA	Late embryogenesis abundant
MAPK	Mitogen-activated protein kinase
MS	Mass spectrometry
NCS	(S)-noroclaurine synthase
PAGE	Polyacrylamide gel electrophoresis
PR	Pathogenesis-related
PR 10	Pathogenesis-related 10
PVDF	Polyvinylidene diflouride
RNase	Ribonuclease
ROS	Reactive oxygen species
RT	Room temperature
SAM	Shoot apical meristem
SDS	Sodium dodecyl sulphate
SOS	Salt overly sensitive

StAR	Stereodogenic acute regulatory protein
START	Stereodogenic acute regulatory protein related lipid transfer
TBP	Tributylphosphine
TBS	Tris-HCL buffered saline
TCA	Tricholoroacetic acid
TMV	Tobacco mosaic virus
ToF	Time of flight
WT	Wild type

1.0 Introduction

Global population is expected to reach over 9 billion by 2050 and will necessitate an increase in current food production. However, the land available for agriculture is constantly on the decline and although arable land area may be increased by the destruction of forests, as it is happening in various parts of the world, the negative consequences of such actions are numerous. Indeed, Zhang et al. (2006) have predicted that if current trends continue, land devoted to agricultural production will increase in developing countries resulting in a net decrease of forested areas and other non-agricultural land over the next 25 years. Therefore, in order to ensure that the growing global population can have adequate food supply, an increase in crop yield using existing arable land base must be achieved. Furthermore, the increasing interest in using agriculture land for non-food applications, such as biofuels, also places importance on optimizing production on the existing agricultural land base. However, increasing crop yields significantly using the existing land base may be challenging, because abiotic stresses such as drought, salinity, temperature extremes as well as diseases and nutrient deficiencies impose yield penalties necessitating development of novel varieties capable of better withstanding abiotic stresses. Among abiotic stresses that affect crop production, salinity is a major one. In fact, predictions are that salinity could affect upwards of 50% of arable land by 2050 (Wang et al. 2003) Therefore, traditional breeding and genetic engineering alongside modifying current agronomic practices to efficiently develop plants that can better tolerate abiotic stresses are needed to increase crop production (Yamaguchi and Blumwald 2005). Furthermore, focusing on sustainable agriculture and the development of high-yielding and high quality agricultural crops can

increase crop performance and halt the current trend of converting non-agricultural land for agricultural use (Zhang et al. 2006).

1.1 Abiotic stress

As mentioned above, abiotic stresses pose threats to agricultural productivity. Throughout the growing season, plants are continuously exposed to a wide variety of abiotic stresses including, drought, salinity, flooding, temperature extremes, heavy metal toxicity, and ultraviolet radiation. Although abiotic stresses such as, drought, salinity, and temperature extremes may seem quite different from each other, they each disrupt water relations within the plant causing osmotic stress and, as a result, often induce similar responses in the plant (Wang et al. 2003).

1.1.2 Abiotic stress response

Perception of stress by the plant and appropriate responses are key factors in determining how plants tolerate abiotic stress. As mentioned above, osmotic stress is a common component of drought, salinity and low temperature stress and, as a result, plant response to these stresses involves numerous and often overlapping pathways that interact with each other in an attempt to combat the deleterious effect of these stresses. For example, expressed sequence tag (EST) analysis by Wong et al. (2005) on *Thellungiella salsuginea* leaves stressed with cold, drought and salt revealed transcripts for genes that deal with reactive oxygen species (ROS) and others involved in transcription or signal transduction. In the same study a MYB family transcription factor was present in all three EST groups

indicating that it may play a general role in regulating the cold, drought and salinity stress response (Wong et al. 2005).

MYB and MYC recognition elements and ABA-responsive elements (ABRE) are regulated by abscisic acid (ABA) to induce the expression of stress responsive genes containing recognition sequences for these elements (reviewed in Wang et al. 2003; Chinnusamy et al. 2005). ABA levels increase in response to osmotic stress in order to regulate water balance within the plant and induce stomatal closure to protect the plant cell from dehydration. Furthermore, ABA also induces *ICK1*, a cyclin–dependent-protein–kinase inhibitor that may inhibit cell division (Wang et al. 1998), an important consideration under abiotic stress, when photosynthesis and carbohydrate production are not optimum.

Although ABA-dependent responses are an important part of abiotic stress responses, ABA-independent pathways also exist. For example, dehydration responsive transcription factors (DREB) and C-repeat binding factors (CBF) both respond to both low temperature and water stress, activating the transcription of several genes driven by the DRE/CRT sequence that are involved in stress tolerance (reviewed in Wang et al. 2003; reviewed in Yamaguchi-Shinozaki and Shinozaki 2006). Over-expression of members of the DREB/CBF family in a variety of plant species has increased tolerance to drought, salinity and freezing stress (reviewed in Yamaguchi-Shinozaki and Shinozaki 2006). Both ABA-dependent and ABA-independent pathways activate signaling cascades that result in the expression of late embryogenesis abundant (LEA) -type proteins, which provide dehydration tolerance, carry out a protective role during osmotic

stress (reviewed in Chinnusamy et al. 2005) and, in the case of freezing stress, induce the same genes that respond to dehydration stress (reviewed in Mahajan and Tuteja 2005).

Calcium also plays a role in abiotic stress signaling and activates Ca^{+}-dependent protein kinases (CDPKs) that have demonstrated involvement in cold (reviewed in Mahajan and Tuteja 2005), drought and wounding signaling pathways (reviewed in Chinnusamy et al. 2004). Furthermore, Ca^{+} plays a role in activating the Salt Overly Sensitive (SOS) pathway in *Arabidopsis* that contributes to maintaining cellular homeostasis (Chinnusamy et al. 2005). Liu and Zhu (1997) reported that a salt overly sensitive 3 (*sos3*) mutant was rescued by external Ca^{+} application, which decreased its overall NaCl sensitivity by maintaining the K^{+}/Na^{+} homeostasis in the cytoplasm.

Since cellular dehydration is the major result of cellular water deficit stresses, plants accumulate osmolytes (carbohydrates, glycine betaine, proline, and polyols) that increase the water potential inside the cell to prevent water loss (Mahajan and Tuteja 2005). Although these compounds perform osmotic adjustment, they provide other functions, such as osmoprotection, free radical scavenging and macromolecule protection (reviewed in Parida and Das 2005). Under conditions of cellular dehydration oxidative stress occurs which induces the production of ROS, such as superoxide, hydrogen peroxide, hydroxyl radical and singlet oxygen (reviewed in Parida and Das 2005). ROS cause the plant to produce antioxidant enzymes, such as glutathione-*S*-transferase, superoxide dismutase (SOD), and peroxidases to combat cellular damage (Wang et al. 2003). However, under environmental stress, the production of antioxident enzymes may not be sufficient and, as a result, ROS may cause oxidative damage in the cell by compromising lipid, protein and nucleic acid function (reviewed in Parida and Das 2005).

In addition, ROS activates mitogen activated protein kinase (MAPK) signaling cascades in *Arabidopsis* that are involved in the abiotic stress response (Chinnusamy et al. 2005) and represent a point where multiple stress signals converge (Chinnusamy et al. 2004).

Furthermore, the synthesis of heat shock proteins (reviewed in Wang et al. 2003) and the expression of pathogenesis-related (PR) proteins (Liu and Ekramoddoullah 2006) are also common components of the plant abitoic stress response. Salinity and low temperature stress, two important cellular water deficit stresses, will be the focus of further discussion.

1.1.3 Effects of salinity stress

Saline soils affect the ability of plants to uptake water. Under saline soil conditions, the low soil water potential, due to increased solute concentration present in these soils, creates an energy gradient that favors the movement of water out of the plant. This difference in water potential between the plant and the soil causes the plant cell to dehydrate as water moves from the cytoplasm to the extracellular space (reviewed in Bartels and Sunkar 2005). Salinity causes stunted growth, reduced shoot and root biomass, decreased yield, reduced leaf expansion due to decreased photosynthesis, and changes in leaf morphology, such as epidermal thickening (reviewed in Parida and Das 2005). The loss of water from the cytoplasm causes osmotic stress in the cell, disrupting water and ion homeostasis (Zhu 2003), while the loss of turgor leads to stomatal closure and reduced photosynthesis (Zhu 2001). Furthermore, Na^+ takes over the transporters and pathways that usually uptake the essential plant nutrient K^+. An excess of Na^+ has a

number of deleterious effects on cell metabolism and overall plant health: disruption of membrane potential and in increased uptake of Cl^- down the chemical gradient, causing leaf chlorosis and necrosis (reviewed in Greenway and Munns 1980); breakdown of cell membrane organization; reduction of cell division and cell expansion; induction of osmotic imbalance; imposition of toxic effects on metabolism; disruption of enzyme function; reduction of photosynthesis and the production of ROS (reviewed in Greenway and Munns 1980; reviewed in Parida and Das 2005).

There are two strategies that plants utilize to deal with salinity stress: plants either exclude salinity related ions from their cells, or they have mechanisms to tolerate them within the cell. This can involve selective uptake or exclusion ions, control of ion uptake and transport throughout the plant, sequestration of ions within the plant, synthesis of osmolytes, and induction of plant hormones or antioxidant enzymes to deal with ROS generated as a result of the stress (reviewed in Parida and Das 2005; Zhu 2001).

1.1.4 Low temperature stress

Plants require a certain temperature range to experience optimum growth, whether this is during germination and early seedling growth, or during maturation and seed set. When plants experience temperatures outside their optimum range a variety of phenotypic and physiological changes can occur. For example, wilting, chlorosis, necrosis, reduced growth and reproductive capabilities are a few of the ways that cold stress can impact plant health and ultimately reduce yield (Mahajan and Tuteja 2005). Like salinity stress, low temperature stress also affects water relations within the plant cell because of ice formation in the apoplast. When ice crystals form in the intercellular space the water

potential decreases and water moves into the intercellular space from the area of high water potential in the cytoplasm (Sharma et al. 2005). Similar to salinity stress, low temperature stress also leads to dehydration in the plant cell; however, low temperature stress can also lead to the enlargement of intercellular ice crystals, putting a physical strain on the cell wall and membrane, leading to cell rupture (Mahajan and Tuteja 2005). Overall, low temperatures damage the cell membrane and impair cell function due to the loss of organelle compartmentalization, disruption of photosynthesis and protein production (Mahajan and Tuteja 2005).

Fortunately plants have the potential to adapt to the lower temperatures experienced during seasonal transitions when plants can be exposed to lower than optimal temperature ranges. Cold acclimation involves numerous physical and biochemical changes that help the plant tolerate the membrane damage and dehydration that come with freezing injury. Membrane fatty acids change from saturated to unsaturated in order to stabilize the cell membrane (Mahajan and Tuteja 2005) and the structures of proteins as well as their flexibility are modified (Sharma et al. 2005).

Both salinity and low temperature stresses impact overall plant productivity and health. They impact stand establishment by affecting germination and seedling growth and also affect yield by reducing plant fitness and reproductive capabilities. The process of germination and plant development, as well as the impact of abiotic stresses on them, will be discussed in subsequent sections.

1.2 Germination

Germination is the transformation of a dehydrated seed with minimal metabolism into a seed with an active metabolism, culminating in a growing and developing seedling (Bewley and Black 1994). Under conditions of sufficient moisture, temperature and oxygen, germination may occur, beginning with the movement of water (imbibition) into the quiescent seed. Imbibition reintroduces water into the dry seed and metabolic activities, such as respiration, transcription, protein synthesis, and repairs to structural and metabolic components damaged during the prior desiccated state resume (Bewley 1997; reviewed in Finch-Savage and Leubner-Metzger 2006). Germination is complete when the radicle emerges, termed visible germination, after which seedling growth commences.

1.2.1 Germination and abiotic stress

As mentioned previously, in order for germination to begin the seed needs to imbibe water and experience suitable environmental conditions. Imbibition is dependant on the water relations between the seed and the soil; water moves from an area of high water potential to an area of low water potential. Therefore, if there is a higher concentration of solutes in the cell than there is in the soil, the cell has lower water potential and water will move from the soil into the cell. For example, Rinaldi et al. (2005) reported that at low soil water potential (-0.8 MPa) wheat seeds took three times longer to germinate when compared to those at higher soil water potential (-0.01 MPa), where germination was always greater that 75%. Therefore, under circumstances where low soil water

potential exists, such as in saline soils or under drought conditions, germination will be hindered.

When imbibition begins, the difference in water potential between the seed and the soil under non-saline conditions is quite large and, in fact, there is a linear relationship between germination rate and water potential (Finch-Savage and Leubner-Metzger 2006). As the seed becomes increasingly hydrated, the movement of water from the soil to the seed decreases over time (Bewley and Black 1994). In situations where the minimum required water potential is not met, germination will not proceed to completion (Finch-Savage and Leubner-Metzger 2006). Soltani et al. (2006) reported that wheat seedlings grown under saline and drought conditions mobilize less of the seed reserves for growth than those grown under normal conditions, indicating that salinity affects the efficiency of germination as well as seedling growth. When the embryo axis expands and elongates to break through the outer covering of the seed, the difference in water potential facilitating this expansion is a result of decreased osmotic potential caused by the production of low molecular weight osmolytes hydrolyzed from stored reserves (Bewley and Black 1994). Saline soil conditions inhibit the elongation of the embryonic axis and subsequent seedling growth due to low pressure potential in the cells. Therefore, osmolytes can impact the success of germination under both normal and saline conditions. For example, Vardhini and Rao (2003) demonstrated that the application of brassinosteroids on sorghum seeds increased germination and seedling growth by increasing soluble proteins and proline in six-day-old seedlings. Sohn et al. (2005) also reported that a rice cultivar demonstrating high germination in the presence of NaCl contained a higher seed proline content when compared to the other cultivar. In addition,

Poljakoff- Mayber et al. (1994) reported that seeds failing to germinate under saline conditions did not demonstrate an increase in proline when compared to their germinated counterparts, supporting an important role for proline during germination of seeds under saline stress conditions. Throughout the process of germination the rate of water uptake into the seed is critical, because if it is too slow, the seeds can deteriorate and if it is too rapid, the seeds can be damaged (Bewley and Black 1994).

Although water relations between the soil and the seed are important, soil temperature is also an important factor in germination. Studies on germination of white spruce (*Picea glauca*) reported that germinating seedlings, where radicle emergence had occurred, demonstrated lower tolerance to freezing stress when compared to imbibed seeds. Therefore, the timing of germination is important, because if frost were experienced soon after germination seedling emergence would be affected (Coursolle et al. 1998).

1.3 Flowering

Another important stage in the development of flowering plants is the transition from vegetative to reproductive growth. In agricultural plants this shift is important, because it can determine whether the plant will produce optimal yields, or fall short of the desired harvest. The transition from the vegetative to the reproductive stage is initiated by two major factors, photoperiodism and vernalization; however, temperature, irradiance, and water availability can also impact the transition to flowering (reviewed in Bernier and Perilleux 2005).

1.3.1 Flowering and abiotic stress

The perception of environmental cues involves internal plant signaling to notify the shoot apical meristem (SAM) if the environmental conditions the plant is experiencing are conducive to flowering (Bernier and Perilleux 2005). For example, in *Sinapis alba* the phloem sap displays an increase in sucrose and cytokinins (CK) when exposed to long days. The presence of these two compounds in the SAM causes the plant to begin initiating the transition to flowering (Bernier et al. 2002). It appears that there are a number of factors involved in the transition from vegetative to reproductive growth and that plants respond to these factors differently. For example, *Lolium temulentum* demonstrates an increase in gibberellins (GAs), primarily GA_5, in the leaves prior to flowering and when GA_5 is applied to the leaves flowering is stimulated (King and Evans 2003). On the other hand, *Arabidopsis* does not contain any GA_5 when plant extracts of flowering induced plants are analyzed (Xu et al. 1997). However, the *Arabidopsis* AGAMOUS-LIKE 20 (*AGL20*), an activator of flowering, sequence and function are conserved in orthologues from three closely related crucifers (Kim et al. 2003). The timing of flowering is an important component in plant developmental regulation, because it is a crucial factor in determining reproductive success in agricultural species. For example, in fall planted crops such as fall rye, winter wheat and winter canola, vernalization ensures the plant does not flower on a warm fall day, but instead over-winters and flowers in the spring.

Abiotic stress conditions also impact the transition from the vegetative stage to the reproductive stage. Studies on *Arabidopsis* report that as growth temperatures decrease flowering time increases. For example, Hasdai et al. (2006) reported that at 22

°C flowering occurred after about 25 days, while at 6 °C flowering occurred after 93 days. Similarly, chilling at 5 °C delayed flowering and reduced yield in flax, while heat stress reduced seed yield (Gusta et al. 1997). Furthermore, under subsequent years of drought *Brassica rapa* descendants flowered earlier than their ancestors, indicating that in adapting to drought conditions *B. rapa* avoids drought and flowers earlier (Franks et al. 2007). The ability for crop plants to flower early under stress conditions is important, because heat stress and water stress in late summer will significantly affect crop yield.

1.4 Approaches to improve abiotic stress tolerance

Abiotic stress and the decline of arable land through intensive agriculture pose a major constraint on agricultural productivity (Yamaguchi and Blumwald 2005). With only 10% of the arable land being classified as under non-stress conditions, 90% of the land where crops are grown is impacted by some form of abiotic stress, likely either salinity, drought, extreme temperatures or a combination of the three (Dita et al. 2006). The race to develop crops with enhanced tolerance to abiotic stress has progressed with the availability of genome sequences for *Arabidopsis*, *Oryza sativa*, and *Medicago truncatula*. Furthermore, the development and improvement of various molecular tools such as molecular marker-assisted breeding, proteomics, transcriptomics and genetic transformation has contributed to an improved understanding of abiotic stress responses and aided in the development more tolerant plants (Dita et al. 2006; Yamaguchi and Blumwald 2005). Traditional breeding is time consuming, operates within the genetic variability of the species involved, and demonstrates difficultly in modifying individual traits. Incontrast, modern biotechnological techniques, such as genetic engineering offer

a more precise strategy to develop plants with specific traits and allows the transfer of genes from different species or organisms. Salinity affects many different components of the plant and developing tolerance to this stress is difficult, because it is a quantitative trait (Quesada et al. 2002). As a result, classical breeding for salinity tolerance has not been successful, whereas genetic engineering strategies have shown considerable promise (Borsani et al. 2003). The use of the above techniques to uncover the players in the abiotic stress response and the use of genetic engineering to modify single (or multiple) genes to increase plant tolerance to abiotic stress has seen some success. Expression of transcription factors, Na^+ antiporters, osmosprotectants, and radical scavenging enzymes represent some of the approaches that have been used to engineer abiotic stress tolerance (Borsani et al. 2003) and some examples are discussed in subsequent sections.

1.4.1 Transcription factors

Overexpression of transcription factors is an effective way to enhance abiotic stress tolerance, because they control a wide number of genes from different stress response pathways (Wang et al. 2003) and, as a result, may provide plants with tolerance to multiple stresses. For example, Kim et al. (2004) overexpressed ABA-responsive binding factor (*ABF3*) using a stress inducible promoter and enhanced freezing, heat, drought and oxidative stress tolerance in transgenic *Arabidopsis*. The constitutive overexpression of *DREB1A* increased tolerance to freezing drought and cold stress, but also caused growth retardation under normal conditions (Liu et al. 1998). However, under a stress inducible promoter the *DREB1A* gene demonstrated enhanced tolerance to

freezing, water and salinity stress in transgenic *Arabidopsis*, without inhibiting growth (Kasuga et al. 1999). In addition, Jaglo-Ottosen et al. (1998) reported that overexpression of the transcriptional activator *CBF1* (C-BOX BINDING FACTOR-1) in *Arabidopsis* induced the expression of a number of cold regulated (*COR)* genes and demonstrated increased freezing tolerance in the transgenic line, while in *B. napus* overexpression of two *CBF/DREB*-like genes (*BNCBF5* and *BNCBF17*) conferred a similar response to cold in transgenic plants (Savitch et al. 2005). These studies indicate the potential that transcription factors have in engineering tolerance to multiple stresses and that they may have similar effects in related species, important factors to consider when attempting to develop stress-tolerant plants.

1.4.2 Ion homeostasis

The regulation of ion homeostasis has been shown to improve salinity tolerance in transgenic plants. The removal of Na^+ from the plant has been engineered through the use of Na^+ antiporters. In both transgenic *Arabidopsis* (Apse et al. 1999) and *B. napus* (Zhang et al. 2001) expression of the *Arabidopsis* vacuolar antiporter *AtNHX1* increased salinity tolerance.

1.4.3 Osmotic protection

The overexpression of osmolytes in order to increase the osmotic potential of the cell and stabilize membrane or macromolecules during both salinity and cold stress has been

demonstrated. For example, transgenic *Arabidopsis* with repressed proline degradation demonstrated freezing tolerance and tolerance to high salinity when compared to the wild type (Nanjo et al. 1999), while transgenic rice with increased trehalose production demonstrated higher photosynthetic rates and a decrease in stress induced photo-oxidative damage (Garg et al. 2002). In addition, Sakamoto and Murata (1998) demonstrated that transgenic rice plants with the acquired ability to synthesize glycine betaine in the chloroplast demonstrated increased tolerance to both salinity and low temperature stresses. Directing the synthesis of osmolytes to the chloroplasts may result in high enough concentrations to impart osmotic adjustment. For example, although salt and freezing tolerance has been engineered into tobacco, potato, and *Arabidopsis* through the production of glycine betaine, the concentration of glycine betaine in tobacco and potato may not have been high enough to provide osmotic adjustment. In contrast the concentration in *Arabidopsis* was much higher, because the synthesis was chloroplast specific (reviewed in Holmberg and Bulow 1998).

1.4.4 Antioxidants

Although the above examples may have been involved in enhancing salinity tolerance due to osmolyte function, Zhu (2001) speculates that in some cases their function in increasing salinity tolerance in the transgenic plants is likely due to their role in scavenging ROS, because in many cases their levels in transgenic plants were too low to impart any osmoregulation. For example, transgenic tobacco accumulated more proline when compared to the wild type and demonstrated lower free radical levels in response to

osmotic stress and tolerance to high salinity (Hong et al. 2000). Two other examples of tolerance to salinity and cold via free radical scavenging also involve tobacco. Transgenic plants expressing cDNA encoding an enzyme with glutathione *S*-transferase (GST) and glutathione peroxidase (GPX) activities grew significantly faster after exposure to chilling and salt stress when compared to the wild type (Roxas et al. 1997), while transgenic plants expressing alfalfa aldose-aldehyde reductase showed increased tolerance to heavy metal, salt and dehydration stress (Oberschall et al. 2000). Further evidence of the importance of free radical scavenging during stress comes from the demonstrated salt tolerance displayed by transgenic plants overexpressing genes that can scavenge ROS, such as superoxide dismutase, ascorbate peroxidase and NPK1 a (MAP) kinase (reviewed in Zhu 2001).

1.4.5 Other approaches

Aside from the three major approaches discussed above, abitoic stress tolerance has also been engineered using the following approaches. Engineering transgenic plants that have the ability to form *cis*-double bonds in saturated fatty acids have achieved enhanced tolerance to low temperature stress; tobacco plants expressing ω-3 fatty acid desaturase gene from *Arabidopsis* demonstrated tolerance to chilling (Kodama et al. 1993), while a cyanobacterium Δ9-desaturase gene enhanced chilling tolerance in transgenic plants when compared to the wild type (Ishizaki-Nishizawa et al. 1996).

Transforming plants to synthesize LEA proteins has also shown promise in providing freezing tolerance (Ilami et al. 1997). For example, rice transformed with the

HVA1 gene from barley demonstrated tolerance and enhanced recovery after drought stress when compared to the wild type (Xu et al. 1996).

PR 10 proteins have also provided abiotic stress tolerance in transgenic plants. The transformation of *B. napus* with pea *PR 10.1* cDNA demonstrated enhanced germination under both normal and saline conditions (Srivastava et al. 2004), while transformation of *Arabidopsis* with pea *ABR17/PR 10.4* cDNA conferred tolerance to salinity and cold during germination and tolerance to low temperatures during the seedling stage (Srivastava et al. 2006b). We review the literature on PR 10 proteins in the subsequent section.

1.5 Pathogenesis-related (PR) 10 Proteins

Plant PR proteins are a 14-member group of proteins classified on the basis of their biological activity and/or amino acid sequence (van Loon 1994) and, as their name suggests, are induced in plants after exposure to abiotic or biotic stress. Members of the PR group of proteins have been classified as peroxidases (PR 9), β-1,3 glucanases (PR 2), endochitanases (PR 3, 4, 8, and 11), thaumatin-like proteins (PR 5), defensins (PR 12), proteinase inhibitors (PR 6), as well as endoproteinases (PR 7) (van Loon and van Strien 1999). The tenth member of this group (PR 10) is a family of small (17 kDa) acidic intracellular proteins, in contrast to the extra-cellular nature of other PR proteins, and was first identified in cultured parsley cells after treatment with a fungal elicitor (Somssich et al. 1988). PR 10 proteins show structural similarities and/or homologies with major latex proteins (Osmark et al. 1998), CK specific binding proteins (CSBPs; Fujimoto et al. 1998), vegetative storage proteins (Richard-Molard et al. 2004), steroid-binding proteins

(Neudecker et al. 2001; Markovic-Housley et al. 2003), ribonucleases (RNase) (Moiseyev et al. 1997), as well as the Bet v 1 (Hoffmann-Sommergruber et al. 1997) and Pru av 1 allergen families (Neudecker et al. 2001). They have been found in a variety of gymnosperms and angiosperms (both monocots and dicots), including pea, potato, birch, pine, apple, peanut, hot pepper, rice, alfalfa, and lily (reviewed in Liu and Ekramoddoullah 2006). These proteins have demonstrated ligand-binding activity (phytosteroids and phytohormones; Gonneau et al. 2001; Mogensen et al. 2002), antifungal activity (Chada and Das 2006) and RNase activity (Moiseyev et al. 1994; Bantignies et al. 2000; Park et al. 2004; Srivastava 2006a; 2007), but their biological function remains elusive.

1.5.1 Abiotic and biotic stress response of PR 10

Numerous studies have reported an increase in PR 10 mRNAs and/or proteins in response to both abiotic and biotic stresses. For instance, PR 10 proteins are up-regulated in response to heavy metal stress (Koistinen et al. 2002), drought (Hashimoto et al. 2004), salinity (Kav et al. 2004; Hashimoto et al. 2004; Moons et al. 1997), cold acclimation and wounding (Lui et al. 2003). They are also induced in response to parasitic higher plants (Borsics and Lados 2002), fungal pathogens (Yu et al. 2000; Jwa et al. 2001), bacterial pathogens (Richard–Molard et al. 2004), viral pathogens (Park et al. 2004), and insects (Broderick et al. 1997).

PR 10 proteins show a wide variety of responses to pathogen attack and in some cases may play a role in plant defense. For example, a chicory vegetable storage protein

with homology to PR 10 proteins displayed increased mRNA and protein levels in plants that were resistant to *Erwinia carotovora* when compared to susceptible lines (Richard-Molard et al. 2004). Infection by nonpathogenic disease agents has also been found to induce PR 10 proteins. For example, in rice inoculated with compatible and incompatible varieties of *Magnaporthe grisea* the *JIOsPR10* transcript was induced earlier in the incompatible interaction. These plants did not develop severe symptoms when compared to the plants inoculated with the compatible type, indicating that JIOsPR10 may respond to specific host-pathogen interactions (Jwa et al. 2001). Lo et al. (1999) demonstrated that inoculating sorghum with a non-pathogen, *Cochliobolus heterostrophus,* or, alternatively, with a sorghum pathogen, *Colletotrichum sublineolum,* resulted in a delay in the response of PR 10 proteins by about 30 hours in the pathogenic interaction. Similarly, Park et al. (2004) reported that a PR 10 transcript from hot pepper (*CaPR10*) was induced by the incompatible interaction with TMV-P_0 (Tobacco Mosaic Virus), but not induced with the compatible interaction. In contrast, when apple was infected with an incompatible and compatible race of *Venturia inaequalis,* PR 10 transcription occurred earlier in the compatible interaction (Poupard et al. 2003). Furthermore, different PR 10 proteins in the same species may respond differently to pathogen infection; McGee et al. (2001) reported that even though two rice PR 10 proteins, RPR10a and RPR10b, were induced after infection with *M. grisea* their expression patterns differed. RPR10a increased within the first 12 hours after inoculation while RPR10b began to increase 48 hours after inoculation (McGee et al. 2001). These studies indicate that PR 10 proteins may play a role in increasing tolerance to pathogens in resistant plant varieties, as well as being part of the plant's response in the incompatible host-pathogen interaction.

A wide variety of abiotic stresses can also cause an increase in PR 10 gene expression in plants. Yu et al. (2000) reported that western white pine (*Pinus monticola* Dougl. ex D. Don) was also shown to accumulate a PR 10 protein, Pin m III, in the winter specifically in the roots and twigs. Similarly, in mulberry (*Morus bombycis* Koidz.), a PR 10/Bet v 1 homolog WAP18 (winter accumulating 18 kDa protein) increased during the winter (Ukaji et al. 2004). Alternatively, dormancy initiated an increase in PR 10 transcripts and a reduction in the same when the plants resumed growth in *Retama raetam,* a desert legume (Pnueli et al. 2002). Wounding is also a common inducer of PR 10 expression (Liu et al. 2003) and numerous authors have also reported salt induced specific PR 10 transcript and/or protein accumulation in rice roots (Hashimoto et al. 2004), rice shoots (Moons et al. 1997), and pea roots (Kav et al. 2004). In addition, zinc was found to up-regulate the PR 10c protein in birch roots (Koistinen et al. 2002). It is apparent form these numerous examples that, like biotic stress, a wide range of abiotic stress conditions affect PR 10 proteins expression.

Furthermore, some PR 10 proteins demonstrate induction by both abiotic and biotic stress. Liu et al (2003) observed that *PmPR10* transcripts in western white pine were induced in response to fungal infection, acclimation to cold and wounding, but a number of the transcripts that responded to wounding displayed a different response to cold acclimation and fungal infection. Hashimoto et al. (2004) reported that a root-specific PR 10 protein was not expressed in response to cold stress, but it was expressed in rice roots after salt, drought, and *Magnaporthe grisea* infection. Although PR 10 proteins respond to both abiotic and biotic stress, it is clear that these proteins show

species-specific and stress-specific responses, making it difficult to characterize the precise roles of PR 10 proteins *in planta*.

1.5.2 Structure and function of PR 10 proteins

Although, as described above, PR 10 proteins show a variety of responses to abiotic and biotic stress, they demonstrate highly conserved amino acid sequence and structure. Structural studies on PR 10 proteins in *Pachyrrhizus erosus* (Wu et al. 2002), yellow lupin (Pasternak et al. 2004), and the Bet v 1 (Biesiadka et al. 2002) and Pru av 1 (Neudecker et al. 2001) allergens demonstrated that an internal cavity and glycine-rich loop are highly conserved in PR 10 proteins across these species. These results are in agreement with similarities between PR 10 proteins at the primary structural level, particularly with respect to the glycine-rich loop which is not only conserved in sequence but also in three dimensional structure (Hoffmann-Sommergruber et al. 1997; Biesiadka et al. 2002). The glycine-rich loop is structurally similar to the phosphate-binding (P-loop) structure that is commonly found in nucleotide–binding proteins, giving structural support for the reported RNase activity of some PR 10 proteins (Hoffmann-Sommergruber et al. 1997). Furthermore, the internal cavity, formed by the folding of a rigid β-sheet around an α-3 helix, may be important for the ligand-binding properties of some PR 10 proteins (Biesiadka et al. 2002). The degree of conservation of the glycine-rich loop and the internal cavity has led researchers to suggest that both of these regions may play important roles in the biological function(s) of PR 10 proteins (Figure 1).

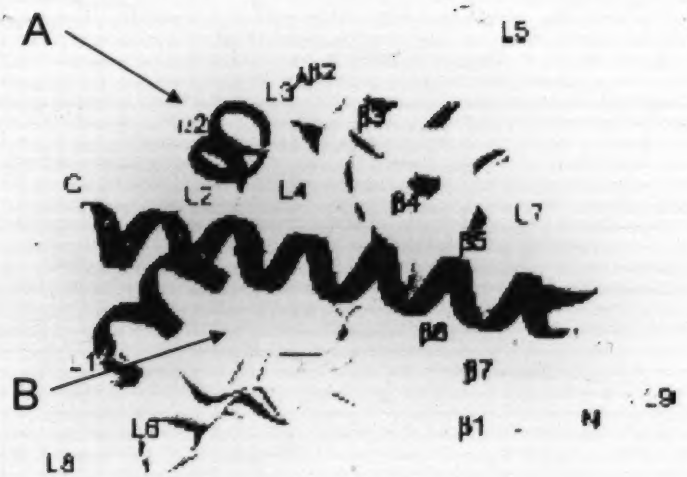

Figure 1 – A ribbon diagram of a PR 10 protein form yellow lupin (LIPR10.1B). The arrows indicate the location of the glycine rich loop (A) and the internal cavity (B). (Biesiadka et al. 2002)

The presence of the glycine-rich loop and the demonstrated RNase activity of some PR 10 proteins and their homologs suggest a possible role for these proteins. Moiseyev et al. (1997) reported that two RNases from ginseng callus cells were homologous to the PR 10 family of proteins and hypothesized that the function of PR 10 proteins may be to cleave intracellular RNA. In addition, RNase activity has been reported in the Bet v 1-Sc1 protein from birch pollen (Swoboda et al. 1996), in the recombinant and native pea PR 10.1 and ABR17/ PR 10.4 proteins (Srivastava et al. 2006a; 2007) and in white lupin root PR 10 (LaPR 10; Bantignies et al. 2000). In addition, CaPR10, isolated from hot pepper (*Capsicum annuum*), demonstrated RNase activity against total *C. annuum* and Tobacco Mosaic Virus (TMV) RNA, anti-viral activity against TMV, and anti-microbial activity against the pathogenic fungus *Phytopthora capsici* (Park et al. 2004). This protein has also been demonstrated to increase in abundance and in its RNase activity when plants were inoculated with TMV. These results suggest that the RNase activity of this PR 10 protein may be involved in protecting the plant from viral and pathogen attack. Furthermore, Koistinen et al. (2002) reported that the PR 10c protein from birch was S-glutathiolated after birch roots are exposed to zinc and maintained RNase activity even after this modulation. This implies that the role of the protein could be in ameliorating oxidative stress damage in the plant. The authors hypothesize that the RNase activity may be involved in increasing the amounts of polynucleotides for increasing transcription needs during the period of stress and that the S-glutathiolation could protect the reactive thiol group and RNase activity from oxidative stress caused by the metal. (*S*)-noroclaurine synthase (NCS), an enzyme involved in benzylisoquinolone alkaloid biosynthesis, shows 28-38% amino acid sequence similarity with PR 10 and Bet v 1

proteins, with speculated structural homology as well, leading the authors to hypothesize that the catalytic function of the NSC enzyme was derived from a PR 10 ancestor possessing RNase activity (Samanani et al. 2004).

The amino acid residues Glu^{97}, Glu^{149} and Tyr^{151} are strictly conserved in PR 10 and are thought to contribute to Bet v 1/PR 10's RNase activity (Moiseyev et al. 1997). For example, Wu et al. (2003) reported that amino acid mutations at Glu^{147} and Tyr^{150} (corresponding to the location of Bet v 1 Glu^{149} and Tyr^{151}) decreased RNase activity in *Pachyrrhizus erosus* PR 10 protein. Although, the purified protein from *Pachyrrhizus erous* demonstrated RNase activity, when the "P-loop" was deleted the RNase activity decreased (Wu et al. 2003). Similarly, mutations of the corresponding amino acids in the GaPR 10 protein from cotton also resulted in the loss of RNase activity, further supporting an important role for these amino acids during catalysis. Additionally, mutations that corresponded to the "P-loop" region of GaPR 10 decreased the RNase activity by half (Zhou et al. 2002). Furthermore, it is interesting to note that the "P-loop" structure of the GaPR 10 protein shows considerable variation from the typical GXGGXGXXK sequence present in other PR 10 proteins with demonstrated RNase activites.

In contrast, Biesiadka et al. (2002) reported that the glycine-rich loop present in many PR 10 proteins is structurally different from the standard "P-loop" structure and therefore, may not be related to PR 10's demonstrated RNase activity, speculating that a more plausible function may be ligand binding. For example, an intracellular CK specific binding protein (CSBP) has homology to PR 10 proteins and the highly homologous Bet v 1 allergen family possesses a high affinity for urea and purine type

CKs (Fugimoto et al. 1998). Similarly, Gonneau et al. (2001) reported that a 34 kDa urea-type CK-binding protein from the moss *Physicomitrella patens,* showing homology with PR 10 proteins and Bet v 1 allergens, was photoaffinity labeled by a cytokinin agonist. As a result, the authors postulate that this protein may be involved in CK binding/signaling or play a role in the CK-mediated stress response. An important study by Mogensen et al. (2002) demonstrated that Bet v 1 binds fatty acids, flavonoids, and CKs, lending support to the ligand binding hypothesis. By studying the displacement of 8-anilino-1-naphthalenesulfonic acid (ANS), a fluorescent probe, the authors were able to demonstrate that the protein binds ANS in its internal cavity. When compared to the other ligands studied, they found that CKs did not displace ANS, but instead appeared to bind at a site close to it because of an increase in ANS fluorescence. This led the authors to hypothesize that CK binds to the glycine-rich loop, because the loop is located in the largest opening to the internal cavity, which would allow CK to bind close to ANS. Explaining the change in fluorescence, Mogensen et al. (2002) speculated that the presence of Bet v 1 in the seed could be involved in CK binding, storage, and release during germination. Furthermore, Markovic-Housley et al. (2003) speculated that the hypoallergenic isoform of Bet v 1 may be involved in transporting phytosteroids, because nanospray ionization mass spectrometry (MS) revealed that a specific non-covalent interaction occurred between Bet v 1 and two brassinosteroids. These authors hypothesized that PR 10 proteins may be involved in transporting hydrophobic ligands to cell wall receptors. In addition, Pru av 1, a major cherry allergen has also been reported to bind phytosteroids (Neudecker et al. 2001). The authors note that both proteins share structural homology with the stereodogenic acute regulatory protein (StAR) related lipid

transfer (START) domain of the human MLN64 protein that is involved in cholesterol transport, although sequence homology is only 8.5%. This structural comparison lends further support to the ligand/ steroid-binding hypothesis for PR 10's function. Furthermore, NMR spectroscopy revealed that the Pru av 1 protein does interact with phytosteroids and the internal cavity can accommodate two such molecules. They hypothesized that the large cavity has an important role in the physiological function of the protein, but whether or not the molecule binds phytosteriods or another ligand *in vivo* remains to be ascertained.

In contrast to the focus on possible RNase and binding activity of PR 10, Pnueli et al. (2002) reported that PR 10 homologous transcripts increased in a desert legume when the plants were entering dormancy and decreased when the plants resumed growth, causing them to speculate that the protein might have functions other than RNase activity. The authors suggest that the high number of polar residues per number of side chains, 40% in PR 10 as compared to 50% in dehydrin, and propose that it may function like a LEA-like protein, shielding other proteins from desiccation during the transition towards lower cell water content during dormancy.

It is clear from the aforementioned research on the PR 10 protein's function that there is considerable debate as to whether the protein is a RNase (despite the numerous studies that have demonstrated RNase activites in many PR 10 proteins), or whether it is involved in CK/steroid binding or functions as a protein protector. Further research is needed to delineate the role of PR 10 proteins in mediating abiotic and biotic stress responses and to precisely characterize their biological activities.

1.6 Objectives

The objective of this study was to characterize a doubled haploid (DH) *B. napus* line constitutively expressing the pea abscisic acid responsive 17 (*ABR17/PR 10.4)* cDNA at various developmental stages and to examine germination of the transgenic lines under abiotic stress conditions, such as salinity and low temperature. Previous work from our laboratory demonstrated increased levels of several PR 10 proteins in the proteome of pea (*Pisum sativum*) roots exposed to salinity stress (Kav et al. 2004). One of the isoforms, PR 10.1, was found to enhance germination and early seedling vigor under both normal and saline conditions in transgenic *B. napus* (var. Westar) constitutively expressing the *PR 10.1* cDNA (Srivastava et al. 2004). Furthermore, pea *ABR17* (*PR 10.4*) cDNA constitutively expressed in *A. thaliana* (ecotype WS) was found to increase germination under salinity and cold, while seedlings demonstrated freezing tolerance, indicating that *ABR17* may provide multiple abiotic stress tolerance (Srivastava et al. 2007).

The significant effect that both PR 10 isoforms had in their respective transgenic plants led us to hypothesize that transgenic DH *B. napus* lines constitutively expressing *ABR17* cDNA may demonstrate enhanced germination and early seedling vigor while grown under normal and abiotic stress conditions. Furthermore, expression of *ABR17* in a canola line used by producers (rather than Westar) may facilitate the testing and release of stress tolerant plants that have a direct application in agriculture. We demonstrate that ABR17 enhances germination under saline and cold conditions and hastens flowering in the transgenic when compared to the wild type (WT). We also discuss the proteome analysis of 2-day old, 14-day old, and 22-day old seedlings in order to identify proteins

that may be influenced by *ABR17* constitutive expression and involved in the early flowering response.

2.0 Materials and Methods

2.1 Plant expression vectors

Pea *ABR17* cDNA (provided by Dr. Trevor Wang, Department of Metabolic Biology, John Innes Centre, UK) was amplified using *ABR17* specific primer sequences (5' – 3') GTGGTCG**AAGCTT**ATGGGTGTCTTTGTTTTTGATGATGAATAC and TATATAG**CTCGAG**TTAGTAACCAGGATTTGCCAAAACGTAACC. The *Hind III* and *Xhol I* restriction enzyme sites are indicated in bold on the forward and reverse primers, respectively. The sequenced *ABR17* gene under control of the CaMV35S promoter and the rbcS3' transcriptional terminator was inserted into pKYLX-71 (Schardl et al. 1987). This vector was used to transform *E. coli* (DH5α) competent cells (Invitrogen, Burlington, ON, Canada). Bacterial colonies containing the gene of interest were selected on 15 μg ml $^{-1}$ tetracycline and screened for the presence of the gene using PCR. *Agrobacterium tumefaciens* strain GV3101, *E. coli* strain HB101 carrying the helper plasmid pRK2013, and the binary vector pKYLX-71 carrying the sequenced *ABR17* gene, were used as the vector system for triparental mating. Transconjugant *A. tumefaciens* was selected on solid medium containing 50 μg ml $^{-1}$ rifampicin, 20 μg ml $^{-1}$ gentamicin and 15 μg ml $^{-1}$ tetracycline. Plasmid DNA was extracted from *A. tumefaciens*, sequenced and analyzed by restriction digestion to ensure that no rearrangements had taken place during the mating procedure. Transconjugant *A.*

tumefaciens was used to transform *Arabidopsis* and the *B. napus* lines that were used in this study.

2.2 Transformation of *B. napus*

DH *B. napus* was transformed using the procedure described by Moloney et al. (1989) at the Alberta Research Council (Vegreville, Alberta, Canada). Embryos from T_1 seeds of successful transformants were screened on kanamycin plates (50 $\mu g\ ml^{-1}$) and those with a 3:1 segregation ratio were used to raise homozygous T_2 lines and for subsequent seed production. The segregation ratio was determined based on the number of green to bleached embryos. Twelve independent transgenic lines contained the *ABR17* construct; from these parental lines (T_1) 18 daughter lines (T_2) were selected for further screening. These 18 transgenic lines were tested for their abilities to germinate on half strength Murashige-Skoog (MS) plates (Murashige and Skoog 1962) solidified with 0.8% agarose, supplemented with 1.5% sucrose, pH 5.7, and containing 250 or 275 mM NaCl. Those with enhanced germination relative to their WT were selected for further screening. The T_1 generations grew and matured under different growth conditions and, as a result, the transgenic lines (T_2) were compared to a WT line sharing common T_1 growth conditions.

2.3 Plant growth and germination experiments

Seeds (WT and transgenic) were surface sterilized by soaking in 70% ethanol for 1 minute and in 20% bleach for 20 minutes, after which they were rinsed four times (5 minutes each) with sterile deionized water. Surface sterilized seeds from WT and transgenic lines (2.8, 2.8.2 (T_3 of 2.8), 3.15 and 9.5) of DH *B. napus* were placed on

Whatman filter paper moistened with 5 ml of sterilized deionized water in Petri dishes and germinated in complete darkness at room temperature (RT; 21 ± 2 °C) in order to compare the appearance of these seedlings. For evaluating the ability of these genotypes to germinate in the presence of NaCl, lower temperatures or both, seeds were germinated and grown on half strength MS plates solidified with 0.8% agarose, supplemented with 1.5% sucrose, pH 5.7 in the presence or absence of 275 mM NaCl. In order to evaluate germination under low temperature stresses, plates containing either 0 or 75 mM NaCl were placed at 5 °C or 10 °C in the presence of light. For all germination experiments, seed germination was recorded daily for 7 days (at RT) or for 14 days (at 5 °C and 10 °C). Seeds were considered to have germinated if radicle emergence had occurred. In all experiments at least 5 plates per treatment and 5 seeds per plate were used and each experiment was repeated at least three times.

In order to make observation on these lines at different growth stages, seeds from the WT and transgenic lines (2.8.2, 3.15 and 9.5) were germinated and grown under greenhouse conditions (22 °C day/18 °C night; 16 hour photoperiod). For root observations, seeds were planted in Turface Athletics™ (100% calcined clay; Profile Products LLC, Ill, USA) and grown for 14 days, whereas for whole plant observations, seeds were planted in Metro Mix ® 290 (vermiculite and peat moss; Grace Horticultural Products, ON, Canada) for 37 days or full maturity. Plants were fertilized once every 2 weeks with 200 ppm Peters ® 20-20-20. All observations were made in at least three different batches of plants planted independently and each batch consisted of at least 5 plants.

2.4 Reverse transcription (RT)-PCR analysis

Total plant RNA was isolated (QIAGEN RNeasy Plant Mini Kit, Qiagen, Mississauga, ON, Canada) from pooled 5 week-old leaf tissue from WT and transgenic plants which were flash-frozen in liquid nitrogen. To ensure the complete removal of DNA prior to RT-PCR an RNase-free DNase treatment of the RNA samples was performed as per manufacturer's instructions. cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) and total RNA (50 ng) as recommended by the manufacturer. PCR reactions were carried out with 2 µl cDNA as the template and pea *ABR17*-specific primer sequences: forward 5'-GTGGTCGAAGCTTATGGGTGT CTTTGTTTTTGATGATGAATAC-3' and reverse 5'-TATATAGCTCGAGTTA GTAACCAGGATTTGCCAAAACGTAACC-3' using a PCR Master Mix (Promega, MD, USA) under the following thermocycling parameters: 94 °C, 2 minutes; 35 cycles for 1 minute, 94 °C; 1 minute, 62 °C; 1 minute, 72 °C; and an extension for 10 minutes, 72 °C. Plant *18s rRNA* was used in these experiments as internal control using forward (5'-CCAGGTC CAGACATAGTAAG-3' and reverse (5'-GTACAAAGGGCAGGGAC GTA -3') primers specific for this gene (Duval et al. 2002). Amplification products were separated on a 1% agarose gel and visualized under UV light after staining with ethidium bromide.

2.5 Quantitative real-time-PCR (qRT-PCR) analysis

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to investigate the relative levels of expression of *ABR17* using the expression of actin gene as the internal control. The sequences of the *ABR17*-specific primers and probes were as

follows: forward; 5'-AAATGGAGGTCCAGGAACCAT-3', reverse; 5'- AGCACAT AGTTGGTTTTTCCATCTT-3', probe; 5'-AGAAGCTATCCATTCTT-3' and, for the amplification of the actin gene, the following primers and probes were used: forward; 5'-GCCATTCAGGCCGTTCTTT-3', reverse; 5'-ATCGAGCACAATACCGGTTGT-3', probe; 5'-TCTATGCCAGTGGTCG-3'. qRT-PCR primer sets were designed using Primer Premier software (Applied Biosystems Inc., CA, USA) to generate amplification products that were approximately 70-80 bp in size and RNA isolation, cDNA preparation was performed as described above. PCR reactions contained 2 µl of cDNA (5x dilution), 5 ρmol of probe, 22.5 ρmol of each primer and 1x TaqMan UniversPCR Master Mix (Roche, NJ, USA). The SNP RT template program was used for real-time quantification and was performed in an ABI prism 7700 Sequence detector (Applied Biosystems). The delta-delta method was used to determine relative expression using the following formula: Relative Expression = $2^{-[\Delta Ct\ sample - \Delta Ct\ control]}$, where Ct is the threshold cycle (Livak and Schmittgen 2001). The relative expression of *ABR17* in the transgenic lines was normalized against expression in the WT which was considered to be 1. The experiment was repeated at least three times.

2.6 Antigen preparation and immunization

Pea *PR 10.4* cDNA was amplified using PR 10.4 specific primer sequences 5'-GTGGTCG**CATATG***GAAAATTTGTACTTTCAAGGT*ATGGGTGTCTTTGTTTTTGAT GATGAATAC-3' and 5'-TATATAG**CTCGAG**TTAGTAACCAGGATTTGCCAAAA CGTAACC-3'. The *Nde* 1 and *Xho* 1 restriction enzyme sites are indicated in bold on the forward and reverse primers, respectively, and the protease site is indicated by italics

in the forward primer only. The amplified product was digested with *Nde* 1 and *Xho* 1 and cloned into pET28a bacterial expression vector (Novagen, WI, USA). This vector was used to transform *E. coli* Rosetta (DE3) expression cells (Invitrogen). Expression of the ABR17 N-terminal poly-histidine-tagged fusion protein was induced according to the manufacturer's instructions (Novagen). The bacterial cells were centrifuged at 9 300g for 10 minutes at 4 °C and the pellet was resusupended in lysis buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 15 mM imidazole, 1% Triton x 100, and complete EDTA-free protease inhibitor cocktail (Roche Inc., USA), pH 7.5.

A SONIC 300 Dismembrator (Artek Systems Corp., NY, USA) was used to lyse the bacterial cells. The cells were sonicated at a setting of 0.8 relative output with 10 cycles of 20 second each with a 1 minute cooling on ice between each cycle and then centrifuged at 4 300g for 15 minutes at 4 °C. The supernatant was applied to Ni-NTA agarose (4 ml) in a glass column (1.0 x 30 cm, 24 ml) previously equilibrated with the lysis buffer and reapplied. The column was washed with 10 resin volumes of wash buffer 1 (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM β-mercaptoethanol, 1% Triton x 100, 10% glycerol, 15 mM imidazole, complete EDTA-free protease inhibitor cocktail, pH 7.5), followed by 10 resin volumes of wash buffer 2 (same as wash buffer 1containing 30 mM imidazole). The protein was then eluted with wash buffer 3 (150 mM imidazole) and dialyzed overnight against 50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1 mM DTT at 4 °C. The dialyzed protein was applied to DEAE sepharose (5ml) in a glass column (1.0 x 30 cm, 24 ml) and equilibrated with the dialysis buffer above and the flow through was reapplied. The column was washed with 1.5 resin volumes of the same buffer after which it was washed with the buffer containing 50mM NaCl and subsequently washed

with 50 mM tris-HCL, pH 8.0, 0.5 mM EDTA, 1mM DTT containing 100mM NaCl. The protein was eluted using the same buffer which now contained 500 mM NaCl and the eluted protein dialyzed against 50 mM Tris-HCL, pH 8.0 overnight at 4 °C.

Antibodies were generated by the Bioscience Animal Services (University of Alberta, Canada) using two white rabbits (Flemish Giant X Lop Eared). They were given intramuscular injections containing 65 µg of ABR17 antigen in Freud's adjuvant in a total volume of 1.4 ml. After the initial priming injection (antigen with Freud's complete adjuvant), a total of 2 additional injections (antigen with Freud's incomplete adjuvant) were given approximately 1 month apart, with final serum collection occurring 26 days later.

2.7 Western blot analysis

Pooled two week-old *B. napus* seedlings (0.2 g) were crushed in liquid N_2 and 500 µl extraction buffer (0.5 M Tris-HCl, pH 6.8; containing 10% glycerol; 10% SDS and 60 mM DTT) was added. The tubes were vortexed and placed for 5 minutes in a water bath at 100 °C. After cooling, a few crystals of protamine sulfate were added and the tubes were incubated at RT for 15 minutes and then centrifuged at 12 500g for 15 minutes. Ice-cold acetone containing 0.07% DTT was added to the supernatant and the tubes were centrifuged once again as described above. The pellets were vacuum dried for 15 minutes and resuspended in 150 µl of 50 mM Tris pH 6.8 containing 0.5% SDS and centrifuged again as described above. The concentration of protein in the samples was determined using a modified Bradford assay (Bradford 1976) and the samples stored at -20 °C.

Proteins (25 µg) were separated by SDS-polyacrylamide gel (15%) electrophoresis (Laemmli 1970) using a vertical slab system (Mini PROTEAN 3, Bio-Rad) at constant 150 V until the dye front reached the bottom of the gel. After electrophoresis, the gel was placed in transfer buffer (48 mM Tris, pH 9.2 containing 39 mM glycine, 20% methanol and 1.3 mM SDS) for 15 minutes and the proteins were subsequently transferred to polyvinylidene difluoride (PVDF) membrane for 25 minutes at 15 V using Trans Blot SD, semi-dry transfer apparatus (Bio-Rad). The PVDF membrane was incubated with TBS (10 mM Tris-HCl, pH 7.5; 150 mM NaCl) containing 5% non-fat skim milk powder for 2 hours and then rinsed with TTBS (TBS containing 0.05% Tween 20) for 10 minutes. The membrane was then incubated for one hour in the primary antibody solution diluted 1:20 000 in TTBS after which it was washed 3 times for 5 minutes each with TTBS and incubated for 1 hour in with the secondary antibody (goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugate; Abcam, MASS, USA) which was diluted 1:10 000 in TTBS. The membrane was washed as described earlier, followed by a 5-minute final wash with TBS. Immunoreactive bands on the membrane were visualized by staining with TMB peroxidase substrate kit (Vector Laboratories Inc., CA, USA) according to manufacturer's instructions.

2.8 Protein extraction and two-dimensional electrophoresis (2-DE)

Pooled tissue (0.3 g) from greenhouse grown WT and transgenic (3.15) 2 day-old, 14 day–old shoot and 22 day–old shoot apex tissues were each crushed to a fine powder in liquid nitrogen. The powder was homogenized in acetone containing 10% (w/v) TCA and 0.07% DTT, transferred to eppendorf tubes, and the volume was adjusted to 1.5 ml

with the same solution. Samples were incubated at -17 °C for 1 hour, centrifuged at 13 000g for 15 minutes and the supernatants discarded. The pellets were washed by resuspending them in ice-cold acetone containing 0.07% DTT and centrifuged as described above. The washing step was repeated five additional times, the pellets were vacuum dried at room temperature for 25 minutes and resuspended in 350 µl Rehydration/Sample buffer (Bio-Rad, ON, Canada); 8 M urea, 2% w/v CHAPS, 40 mM DTT, 0.2% Bio-lyte 3-10 and 3.5 µl of 200 mM tributylphosphine (TBP). The samples were vortexed, incubated overnight at 4 °C and centrifuged at 4 °C for 15 minutes at 13 000g. Protein concentrations were determined using a modified Bradford assay (Bio-Rad) with BSA as the standard.

Immobilized pH gradient (IPG) strips (pH 4-7, 17 cm, Bio-Rad) were passively hydrated overnight with 500 µg of protein in 300 µl of Rehydration buffer. Isoelectric focusing (PROTEAN IEF unit, Bio-Rad) was performed under the following conditions using the preset linear program: 15 minutes at 500 volts, 10 000 volts for 60 000 VH, and a 500 volt hold at 17 °C. After first dimension separation, the strips were equilibrated twice in a solution (5 ml/ strip) containing 6 M urea, 2% sodium dodecyl sulfate (SDS), 0.375 M Tris-HCl, pH 8.8, 20% glycerol, and 130 mM DTT for 10 minutes each. The strips were then incubated in the same buffer containing 135 mM iodoacetamide instead of DTT twice for 10 minutes each. Second dimension separation was performed on SDS-polyacrylamide (13%) gels (20 X 20 cm, 1 mm thickness) (PROTEAN II XI system, Bio-Rad) at constant voltage (90 V). Separation was stopped when the dye front reached the bottom of the gel. The gel was stained to visualize the separated proteins using a Colloidal Blue Staining Kit (Invitrogen, CA, USA) according to the instructions

provided. 2-DE was performed at least twice with each of 3 extracted protein samples for a total of 6 gels per genotype.

2.9 Image analysis

Images of 2-D gels were acquired using the GS-800 calibrated densitometer (Bio-Rad) and these images were analyzed using the PDQuest software (Bio-Rad). Six gels each of WT and transgenic (3.15) samples were used to generate the match-sets and individual spots were matched using automated detection tools, with manual verification. Protein spots whose levels were significantly different in the transgenic line were identified using Student's *t*-test feature of PDQuest software as described by the manufacturer. Proteins were considered being up- or down- regulated if they represented a two-fold change or greater from the control. The spots that were reproducibly altered and demonstrated significance ($P < 0.05$) in all three replicates were excised from the gels and subjected to ESI-Q-ToF MS/MS analysis.

2.10 Protein analysis by ESI-Q-ToF MS/MS

Protein spots of interest were excised using a sterile scalpel and placed in a microtiter plate containing 40 µl of sterile water. The gel pieces were processed as described below on a MassPrep Station (Micromass, UK) by the Institute for Biomolecular Design (IBD), University of Alberta, Canada. Excised gel pieces were de-stained and reduced with 10 mM DTT after which they were alkylated with 55 mM iodoacetamide and digested with 6 ng μl^{-1} trypsin (Promega Madison, WI, U.S.A.) in 25 µl of 50mM ammonium bicarbonate for 5 hours at 37 °C. LC/MS/MS analysis of the trypsin-digested peptides

was carried out on a Micromass Q-ToF-2 mass spectrometer (Micromass, Manchester, UK) with a Waters CapLC capillary HPLC (Waters Corp., U.S.A) as described earlier (Yajima and Kav 2006). Briefly, peptides were desalted on a 300 μ x 5 mm PepMap C18 column (LC Packings, California, USA) and separated on a PicoFrit capillary reversed-phase column (5 μ BioBasic C18, 300 Angstrom pore size, 75 μ ID x 10 cm, 15 μ tip (New Objectives, MA, USA) using a linear water/acetonitrile gradient (0.2% Formic acid). Electrospray ionization was used to introduce the eluent to the mass spectrometer. The MS/MS acquisition was performed on detected peptides with a charge state of 2 or 3. The information generated by MS/MS was compared against Sequence Databases with the Mascot search engine (Matrix Science) using the following search parameters: carbamidomethylation of cysteine, possible oxidation of methionine and one missed cleavage per peptide.

2.11 Statistical analysis

For statistical analysis, analysis of variance was conducted by using proc mixed SAS (version 8e, Statistical Analysis System 1985) where genotype was considered as fixed effect and replicate was considered as random effect for all plant characteristics (germination, root and shoot length, height and flowering). The values presented in the graphs are based on the LS means estimates.

3.0 Results and discussion

3.1 Generation of transgenic *B. napus* and confirmation of gene expression

Transgenic DH *B. napus* plants were generated according to Moloney et al. (1989). Plants containing one copy of *ABR17* were chosen based on their segregation ratios on kanomycin plates. Seeds from 18 transgenic lines containing a single copy of *ABR17* cDNA were screened on half strength MS media containing 250 mM NaCl. Three lines, showing the best relative germination (2.8, 3.15 and 9.5), were selected for further studies and homozygous T_2 seeds were used for all experiments (when 2.8 seed stock was depleted a T_3 generation was used, line 2.8.2). Transgene expression was confirmed using reverse transcriptase-polymerase chain reaction (RT-PCR) with pea *ABR17*-specific primers with *18s rRNA* gene as the internal control. The 474 bp amplification product of *ABR17* verified expression in the transgenic lines and was absent in the WT whereas the amplification of the *18s rRNA* gene was evident in both WT and transgenic plants (Figure 3.1A). The presence of pea ABR17 protein was also confirmed in these transgenic plants by Western blot analysis (Figure 3.1B) which revealed an immunoreactive band at the expected molecular weight (~16-17 kDa) in both transgenic lines but not in the WT (Figure 3.1B). Results from these experiments confirmed that the pea *ABR17* cDNA is integrated, transcribed and translated in *B. napus*.

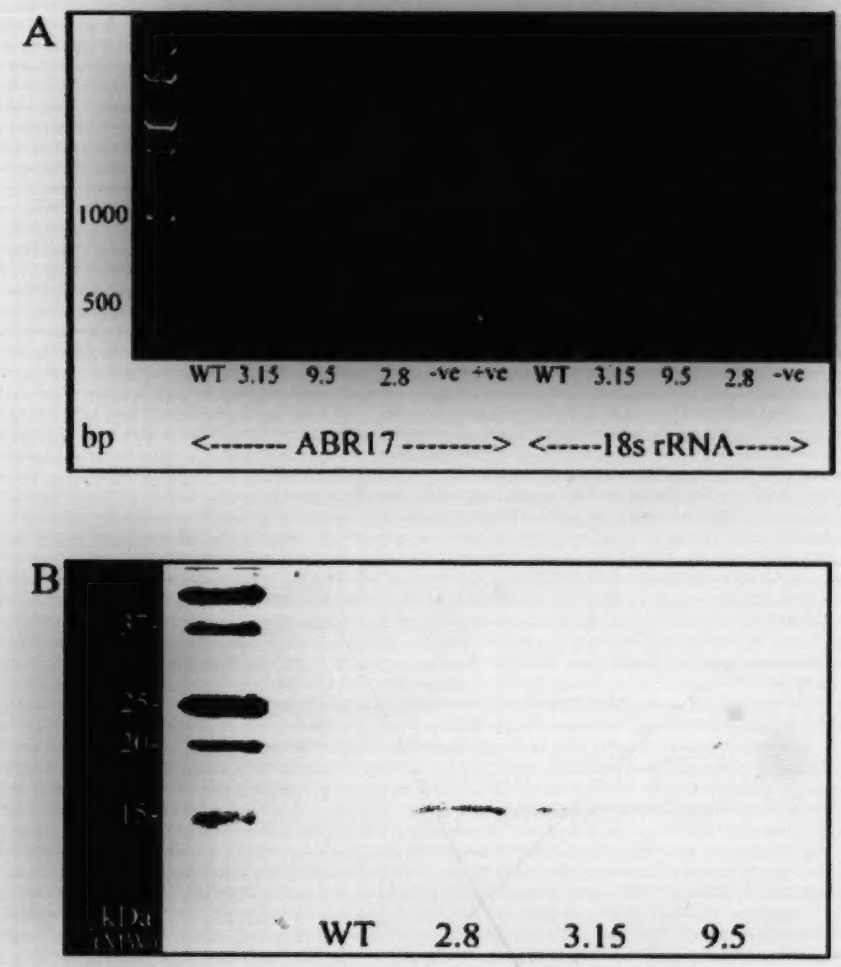

Figure 3.1 - Expression of *ABR17* cDNA in transgenic *B. napus.* Analysis of *ABR17* and *18s rRNA* expression by RT-PCR . The presence of *ABR17* transcript in the transgenic lines (2.8, 3.15 and 9.5) and its absence in the WT as well as *18s rRNA* transcript in both lines is visualized by staining with ethidium bromide (A). Analysis of protein extracts from two-week old seedlings by Western blot. The presence of a unique immunoreactive band in all transgenic lines at the expected molecular weight indicates the presence of ABR17 (B).

3.2 Appearance of *ABR17* transgenic *B. napus* seedlings and plants

The appearance of WT and transgenic seedlings (2.8.2, 3.15 and 9.5) grown on filter paper in sterile water for 7 days and in clay growth medium for 14 days did not demonstrate any visible difference at these stages (Figures 3.2A, 3.2B, 3.2C and 3.2D). Within the WT and transgenic lines there was a variation in root length and shoot length at both stages, but there was no distinct difference between the lines. At 37 days after planting (DAP) plants grown in soil under greenhouse conditions started exhibiting differences between the WT and transgenic line (Figures 3.3A, 3.3B and 3.3C). Transgenic line 3.15 had a longer internode length and more advanced floral bud formation when compared to WT 20. Similarly, transgenic line 2.8.2 was more advanced than the WT, with initiation of internode elongation and advanced floral bud formation, albeit not as advanced as 3.15. Transgenic line 9.5 did not demonstrate any difference from its WT at this stage. At 42 DAP there was a visual difference between the WT lines and transgenic lines 2.8.2, 3.15 and 9.5 (Figures 3.9A, 3.9B, and 3.9C). All transgenic lines were developmentally ahead of their respective WTs and had a greater number of flowers per plant.

3.3 Effects of salinity and low temperature stresses on germination of ABR17-transgenic *B. napus*

The ability of the transgenic lines to germinate in the presence of 275 mM NaCl was evaluated at room temperature (RT; 21 ± 2 °C) and the results are presented in Figures 3.4A-3.4C. The transgenic line 3.15 demonstrated significantly (P <0.05) higher percent germination on all days except days 1 and 7 with the germination on day 3 being

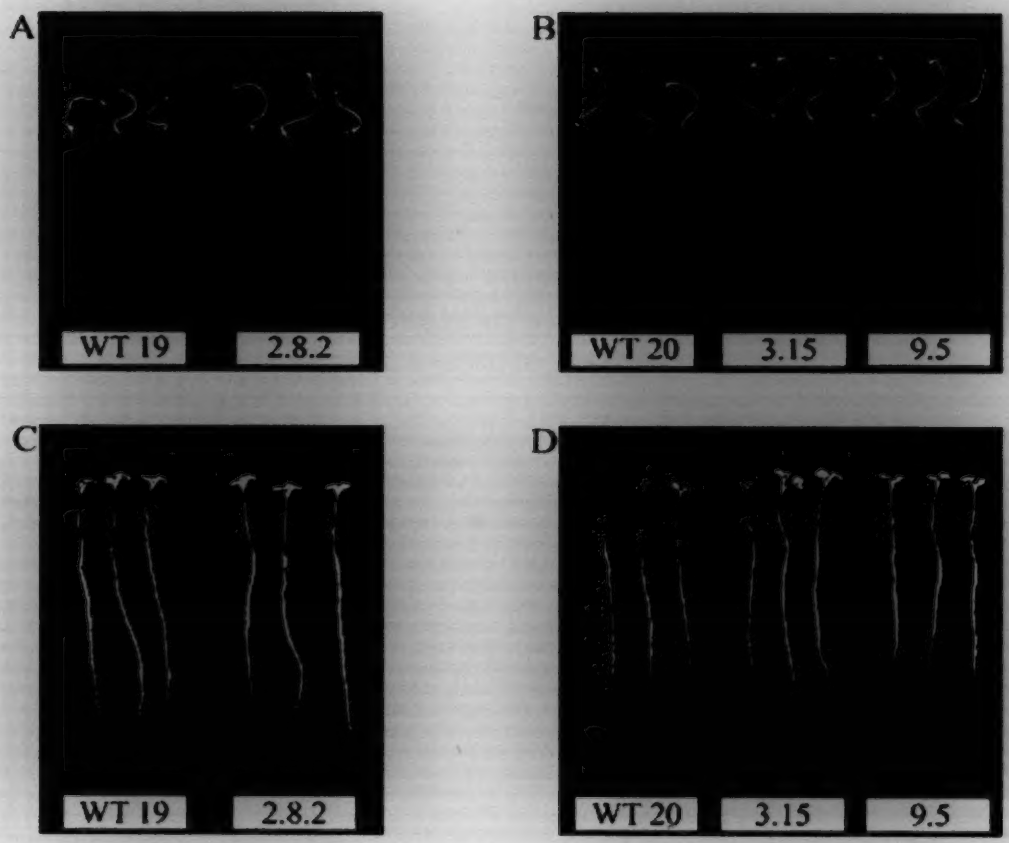

Figure 3.2 – Appearance of WT and *ABR17* transgenic *B. napus* seedlings and plants. Appearance of 7-day (A and B) and 14-day-old seedlings (C and D). Three replicates with a total of 5 plants per replicate were used.

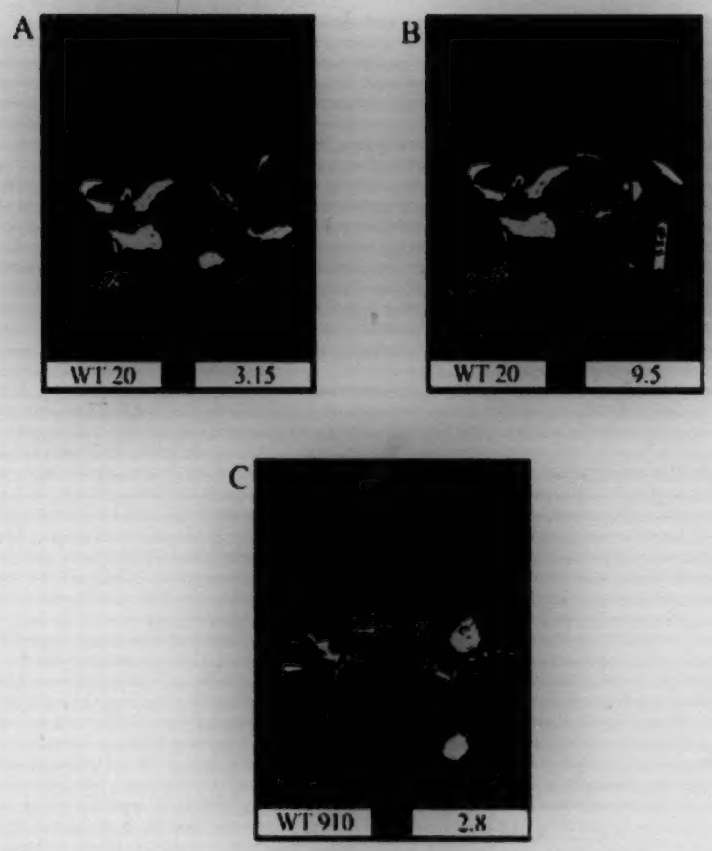

Figure 3.3 - Appearance of WT and *ABR17* transgenic *B. napus* plants 37 DAP. Three replicates with a total of 5 plants per replicate were used.

more than 25% higher in this transgenic line compared to WT 20 (Figure 3.4A). Although not significant, on day 1 line 3.15 had started germinating while lines 9.5 and WT 20 had not. Furthermore, line 3.15 showed significantly longer shoot and root length when compared to WT 20 and line 9.5, indicating that germination as well as initial emergence and growth was greater in line 3.15 during the first seven days compared to the other lines (Figures 3.5A and 3.5B). Lines 2.8 and 2.8.2 did not demonstrate significantly greater germination than their respective WT at 275mM NaCl, nor was there any significant difference in root and shoot length (Figures 3.4B, 3.4C, 3.5C, and 3.5D), in fact line 2.8.2 and WT 19 did not demonstrate any radicle elongation at this NaCl concentration after visible germination had occurred (data not shown).

We have reported previously that the expression of pea ABR17 in *A. thaliana* enhanced the germination of that species in the presence of salt as well as low temperature stresses (Srivastava et al. 2006b). Therefore, we evaluated the ability of *ABR17*-transgenic *B. napus* and WT seeds to germinate at 5 °C and 10 °C alone, as well as in combination with these low temperature stresses and salinity. Once again, at 5 °C, line 3.15 showed significantly greater germination than WT 20 on day 1 (Figure 3.6A). Although line 9.5 was not significantly ahead of WT 20 at this time, it had started germinating while the WT had not. By day two the trend in greater germination demonstrated by line 9.5 on day 1 had become significant. On day 2, 3, and 4, both lines 3.15 and 9.5 were significantly ahead of the WT. In fact, by the third day, transgenic lines 3.15 and 9.5 demonstrated between 5 and 10 % greater germination, respectively, than the WT. When comparing the germination of line 2.8 with WT 910 there was no significant difference in germination between the two lines for the first 5 days. On day 6,

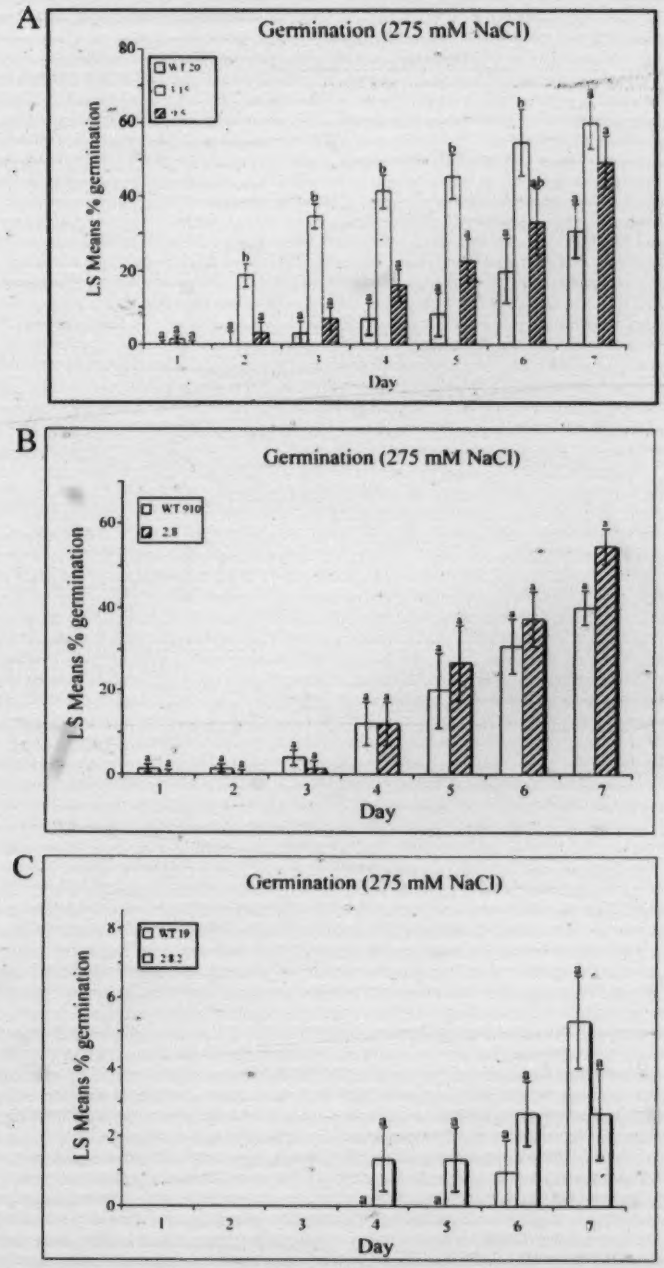

Figure 3.4 - Effect of NaCl on germination of WT and *ABR17* transgenic *B. napus*. Effect of 275mM NaCl on germination of WT20, 3.15 and 9.5(A); WT 910 and 2.8 (B); and WT 19 and 2.8.2 (C). At least three replicates were used with 25 seeds per replicate ($P < 0.05$)

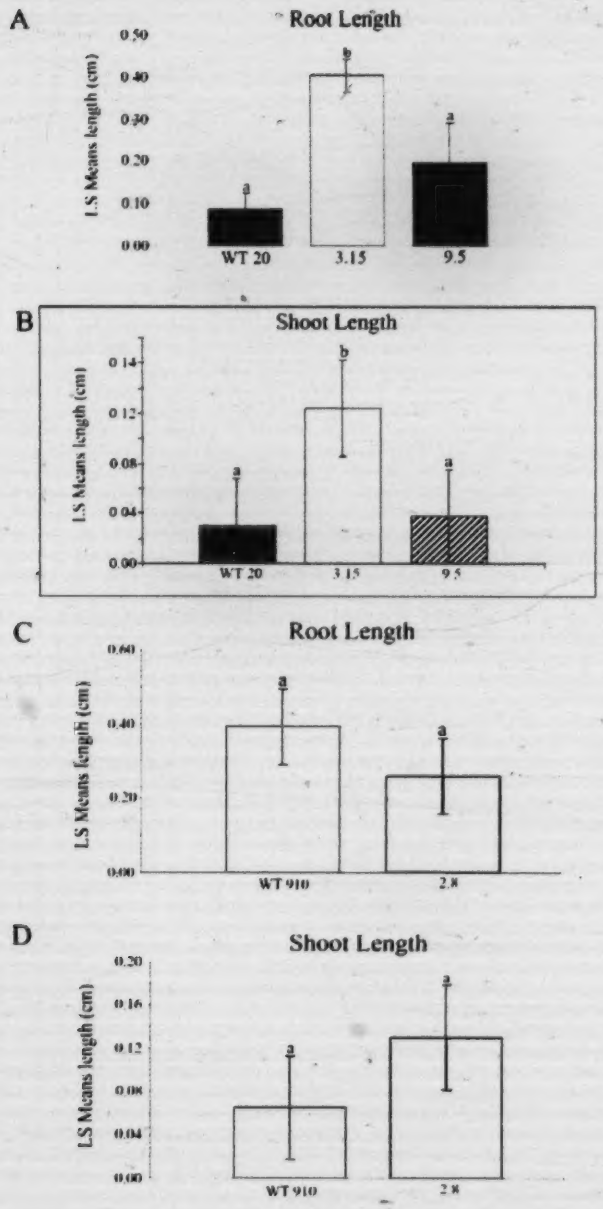

Figure 3.5 - Effect of NaCl on root length and shoot length of WT and *ABR17* transgenic *B. napus* 7 day-old seedlings. Effect of 275mM NaCl on root (A) and shoot length (B) of WT 20, 3.15, and 9.5; root length (C) and shoot length (D) of WT 910 and 2.8. At least three replicates were used with a total of 25 seeds per replicate ($P < 0.05$).

line 2.8 was significantly ahead of the WT and on day 7 line 2.8 showed a trend in greater germination (Figure 3.6B). At 10 °C, although there were no significant differences in percent germination between the transgenic lines that exhibited higher germination at 5 °C (2.8, 3.15 and 9.5), the transgenic line 3.15 displayed a similar trend of increased germination on days 1-3 (Figure 3.7A). Line 2.8 also demonstrated a trend in increased germination when compared to WT 910, but it demonstrated a trend in increased germination towards the end of the observations on days 4 and 12, rather than at the beginning of the observation as was the case with line 3.15 (Figure 3.7B). In addition to investigating the ability of *ABR17*-transgenic seeds to germinate at the lower temperatures described above, we also investigated the effects of a combination of lower temperature and NaCl on the ability of these lines to germinate by testing the effect of 75 mM NaCl on the ability of *B. napus* to germinate at 5 °C and 10 °C. At 5 °C + 75 mM NaCl, germination did not occur in any of the WT or transgenic lines (data not shown). At 10 °C + 75 mM NaCl, significantly ($P > 0.05$) more seeds of the transgenic line 9.5 germinated than WT 20 on days 8 and 12 (Figure 3.8A). Line 2.8.2 demonstrated a trend in increased germination on days 8, 9, and 11 when compared to WT 19 (Figure 3.8B), while line 3.15 did not demonstrate any significant difference in germination from WT 20 (Figure 3.8A). It is unclear why line 9.5 performed better than line 3.15 under these conditions when it was poor in other conditions tested, although positional effects of transgene integration may account for some of the observed differences between the transgenic lines.

Salt tolerance in the *Brassicas* has been associated with ion exclusion. Salt exposed *B. napus* plants have greater K^+/Na^+ ratios in their tissues than other species

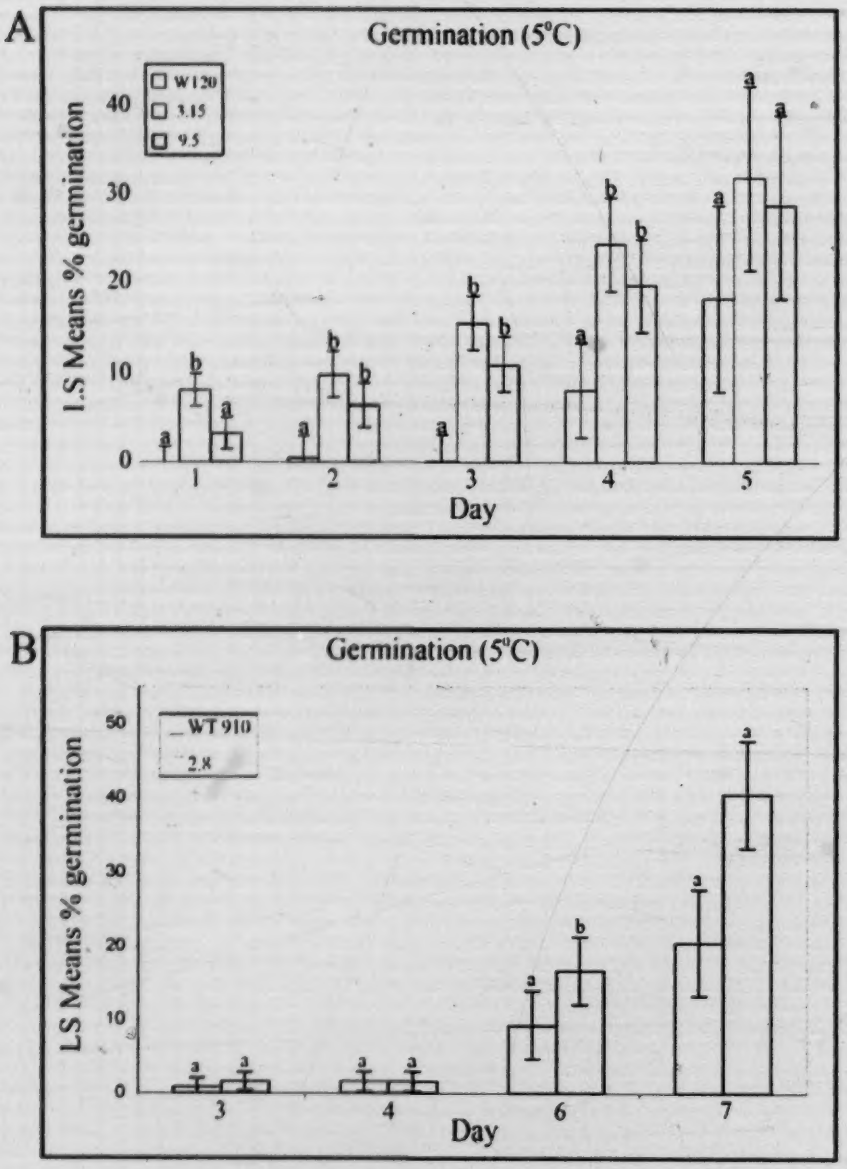

Figure 3.6 - Effect of 5 °C on the germination of WT and *ABR17* transgenic B. *napus* seedlings. Germination of WT 20, 3.15 and 9.5 (A) and WT 910 and 2.8 (B). At least three replicates were used with a total 25 seeds per replicate ($P < 0.05$).

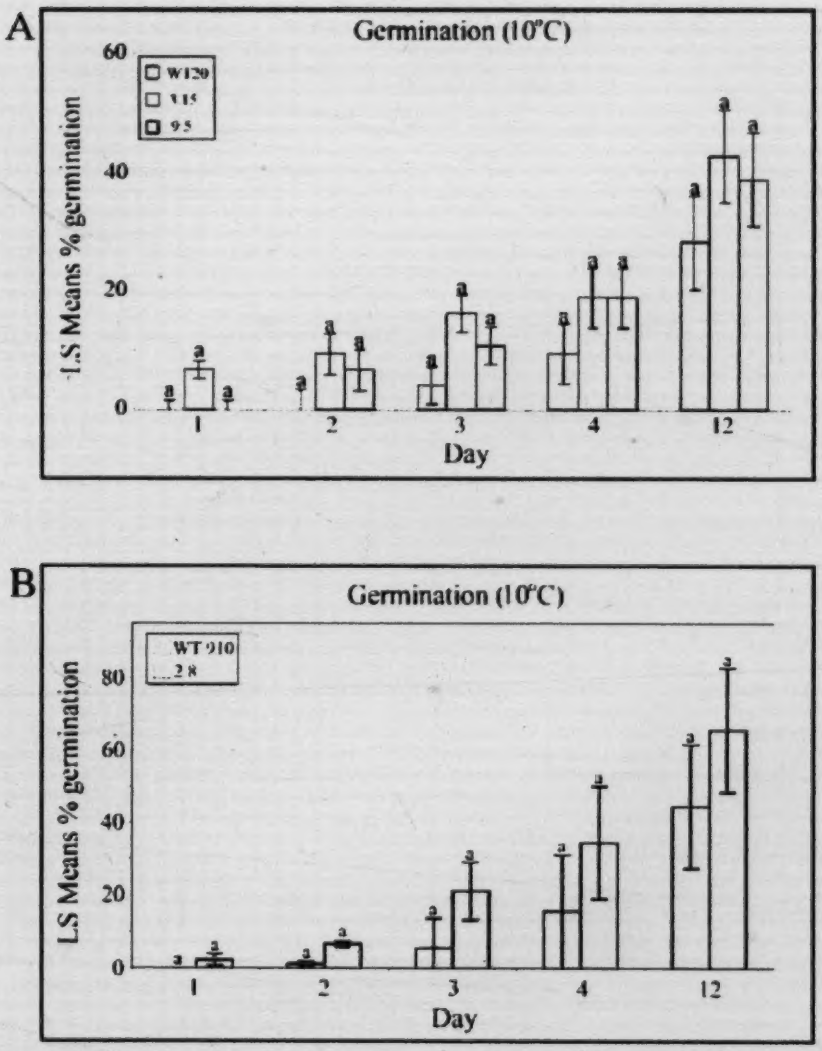

Figure 3.7 - Effect of 10 °C on the germination of WT and *ABR17* transgenic B. *napus* seedlings. Germination of WT 20, 3.15 and 9.5 (A) and WT 910 and 2.8 (B). At leaset three replicates were used with a total of 25 seeds per replicate ($P < 0.05$).

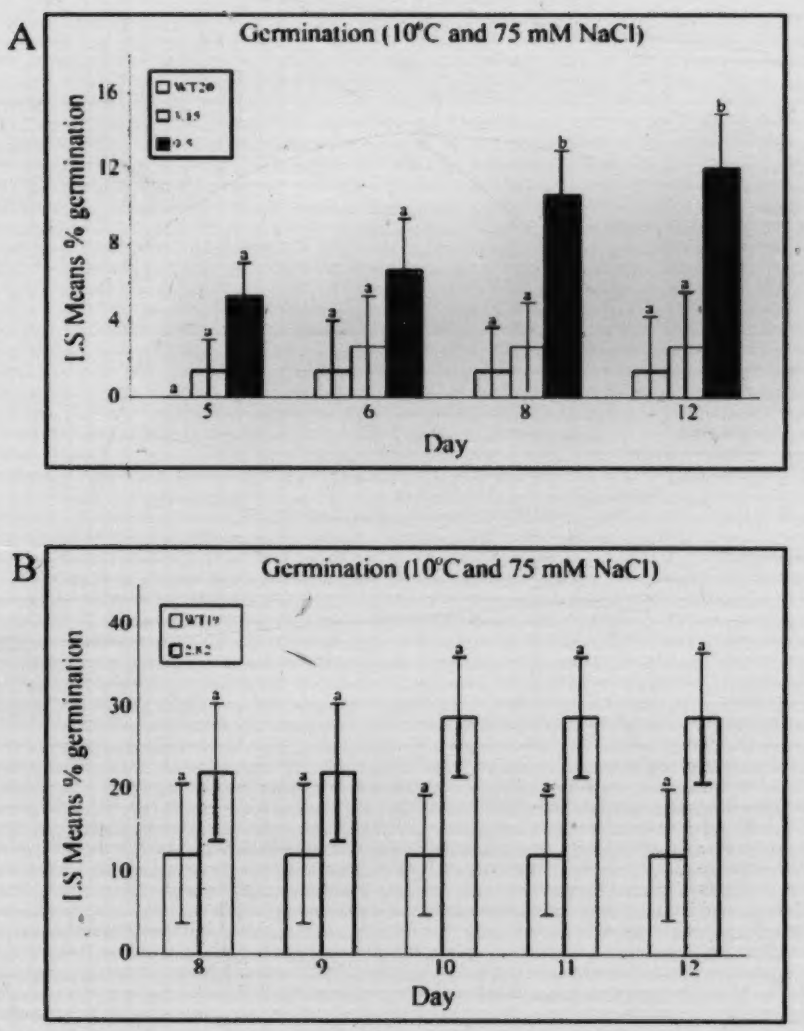

Figure 3.8 - Effect of 10 °C + 75 mM NaCl on the germination of WT and *ABR17* transgenic *B. napus* seedlings. Germination of WT 20, 3.15, and 9.5 (A) and WT 19 and 2.8.2 (B). At least three replicates were used with a total of 25 seeds per replicate ($P < 0.05$).

indicating that they are able to preferentially accumulate K^+ over Na^+, avoiding much of the nutritional stress associated with increased Na^+ uptake under saline conditions (Ashraf and McNeily 2004). The documented tolerance exhibited by *B. napus* (Steppuhn and Raney 2005) was evident in the DH line we were testing, as salinity tolerance during germination (radicle emergence) was observed up to 200 mM NaCl in the WT and transgenic lines 2.8, 3.15, and 9.5 constitutively expressing *ABR17* (data not shown). However, line 3.15 demonstrated increased germination at 275 mM NaCl when compared to WT 20. Generally, low levels of salinity will slow germination down, but not affect the final number of seedlings that germinate, while at higher levels of salinity, the speed of germination will be further reduced with overall germination percentage also decreasing (Bernstein and Hayward 1958). WT 20 and the transgenic line 9.5 demonstrated reduced germination, while the high performing transgenic line 3.15 was ahead in germination after 48 h and continued to be ahead for the first five days after seeding. The increased germination at 275 mM NaCl indicates that the transgenic line does show enhanced germination under saline conditions. Although root growth does not show the effects of salt stress as much as shoot growth (Munns 2002), line 3.15 had significantly greater root growth than line 9.5 and WT 20. Shoot growth in transgenic line 3.15 was also significantly higher than the WT. The greater root length in 3.15 may give this line the added benefit that rapidly dividing root cells have in providing more area for salt sequestration (Bartels and Sunkar 2005) limiting the concentration of salt in the plant (Munns 2002). Furthermore, as the seedlings mature a higher root to shoot ratio ultimately results in better exploitation of soil resources (Pasternak 1987).

Although seedling stages are often regarded as the most salt sensitive growth stage, Bernstein and Hayward (1958) state that poor emergence in saline soils may be a result of soil in the upper portion of the field being more saline, due to evapotranspiration, field morphology and low moisture, rather than low tolerance. Theoretically, in the same soil, the adult plant could avoid the saline regions of the soil profile with their more extensive root system and appear more tolerant. As a result, using one stage of growth to determine the salt tolerance of a specific crop may not be the best approach in determining if a plant is indeed salt tolerant. The early germination seen in our transgenic lines is important, because as Steppuhn and Raney (2005) demonstrated germination and early seedling growth are important parameters in determining the future success of canola under saline soil conditions. They demonstrated that the canola varieties used in their study were comparable to salt tolerant Harrington barley if they displayed good emergence (Steppuhn and Raney 2005). It is important to determine salt tolerance during early growth stages, so that we can establish initial salt tolerance and it will be equally important in future studies to evaluate the performance of the transgenic line as it matures under saline conditions.

Low temperature stress, like salinity stress, impacts crop productivity. Cold temperatures during germination can delay the onset of germination and reduce crop yields; however, the mechanism of how low temperature impacts germination is poorly understood (Salaita et al. 2005). There have been numerous studies on the impact of low temperature stress on germination in tomato (reviewed in Salatia et al. 2005), *Arabidopsis* (Srivastava et al. 2006b; Salaita et al. 2005) and other plants. Cold temperature studies have been performed on *Arabidopsis* seedlings and a number of *COR* genes have been

found to be involved in cold tolerance that encode LEA–like proteins (reviewed in Mahajan and Tuteja 2005) which are synthesized in the seedlings in response to dehydration stress. The promoter elements of *COR* genes contain DRE (dehydration responsive elements) or CRT (C-repeats) and some of them contain ABRE (ABA-responsive element) (reviewed in Yamaguchi-Shinozaki and Shinozaki 2006). The ability to germinate under cold conditions is important in the northern hemisphere, because cold temperatures in the spring may negatively affect crop germination, early seedling growth, and, ultimately, stand establishment. Furthermore, the short growing season and moisture limitations later in the growing season, particularly in the prairie regions, make it important to seed canola crops early so they are able to reach maturity without experiencing a reduction in yield (Angadi et al. 2004). Enhanced germination under salt and cold due to overexpression of *ABR17* cDNA may indicate that molecular responses common to both stresses are responsible for the observed effects mediated by *ABR17*. Our observation that the constitutive expression of pea *ABR17* cDNA in *B. napus* enhances its germination under cold as well as saline conditions may have utility in improving the ability of crop species to germinate and grow under marginal conditions.

3.4 ABR17 expression leads to early flowering in *B. napus*

The transgenic lines tested in this study exhibited dramatic differences in development when visually compared to the WT at 42 DAP (Figures 3.9A, 3.9B and 3.9C), specifically with regard to number of plants flowering and the amount of flowers per plant. The transgenic lines 2.8.2 and 3.15 flowered significantly ($P < 0.05$) earlier when compared to their respective WT line (Figures 3.10A and 3.10B). Furthermore, line 3.15

displayed a significantly faster rate of plants flowering per day when compared to line 9.5 and WT 20 (Figure 3.11A). However, line 2.8.2 did not demonstrate a faster rate of flowering when compared to WT 19 (Figure 3.11B). At 42 DAP, lines 2.8.2 and 3.15 (Figures 3.12A and 3.12B) were significantly taller than the WT. When we examined the genotype effect on average number of days to reach first flower we noticed, as mentioned above, that both 3.15 and 2.8.2 flowered significantly earlier than the WT. Since the transgene had a significant effect on plant height and flowering, we hypothesized that the early flowering observed in the transgenic plants might be due to the effect of height. In order to test this hypothesis we conducted an analysis of covariance on flowering as dependent variable with proc mixed (SAS) where genotype was considered as fixed effect, replicate was considered as random effect and height was considered a covariant. When the effect of height was removed from the analysis, it was apparent that the height effect contributed to the difference in flowering time between line 2.8.2 and the WT. In contrast, height did not contribute to the difference in flowering time observed between the WT and line 3.15. A possible explanation is that the separate transformation events experienced by each line resulted in ABR17 affecting flowering by different factors. In the case of line 9.5, a possible reason for the lack of significant early flowering by line could be that the transgene was integrated in different locations on the *B. napus* genome leading to differential expression which affected its ability to flower earlier.

Similar to germination, flowering is an important developmental process that contributes to determining crop productivity. The timing of flowering becomes extremely important in northern latitudes because of the shorter growing season, which reduces the time for reproductive growth. This can be the case in Alberta, especially the

Figure 3.9 - Appearance of 42 day-old WT and *ABR17* transgenic *B. napus* demonstrating early flowering (A, B, and C).

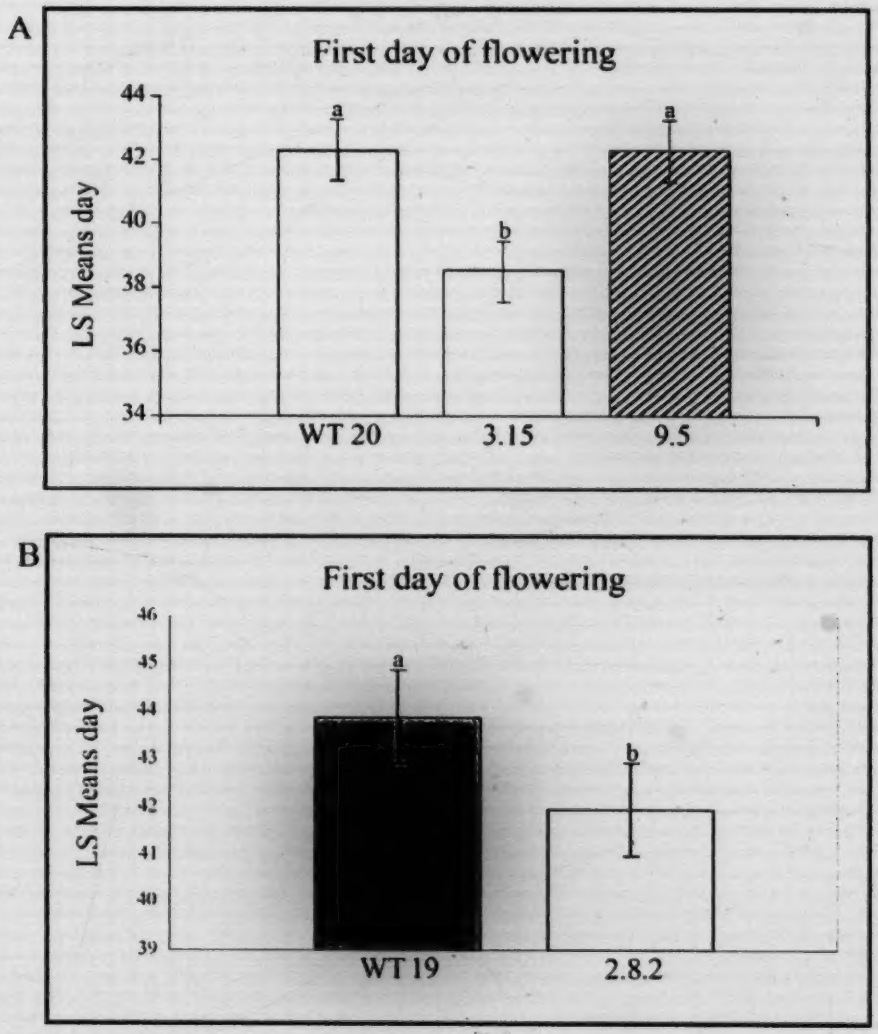

Figure 3.10 – First day of flowering of *ABR17* transgenic and WT *B. napus* adult plant. First day of flowering in WT 20, 3.15 and 9.5 (A) and WT 19 and 2.8.2 (B). Three replicates were used with a total of 10 plants per replicate ($P < 0.05$).

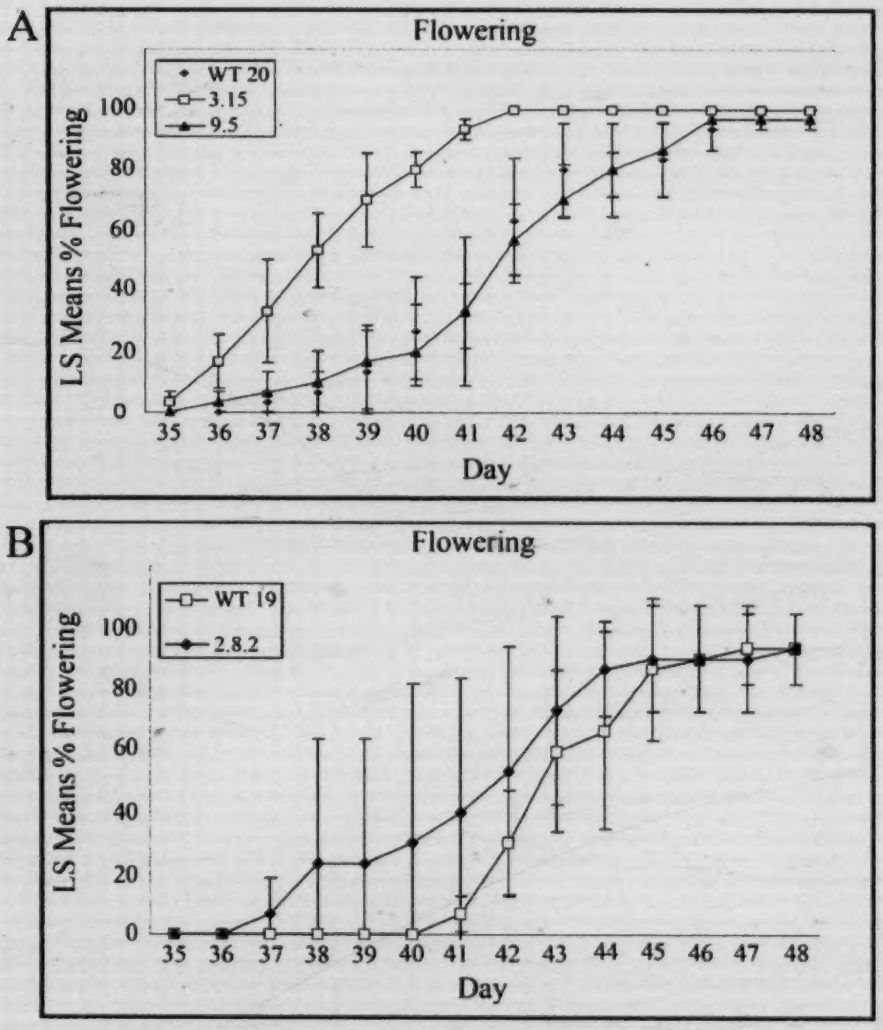

Figure 3.11 – Rate of flowering of *ABR17* transgenic and WT *B. napus* adult plants. Rate of flowering of WT 20, 3.15, and 9.5 (A) and WT 19 and 2.8.2 (B). Three replicates were used with a total of 10 plants per replicate ($P < 0.05$).

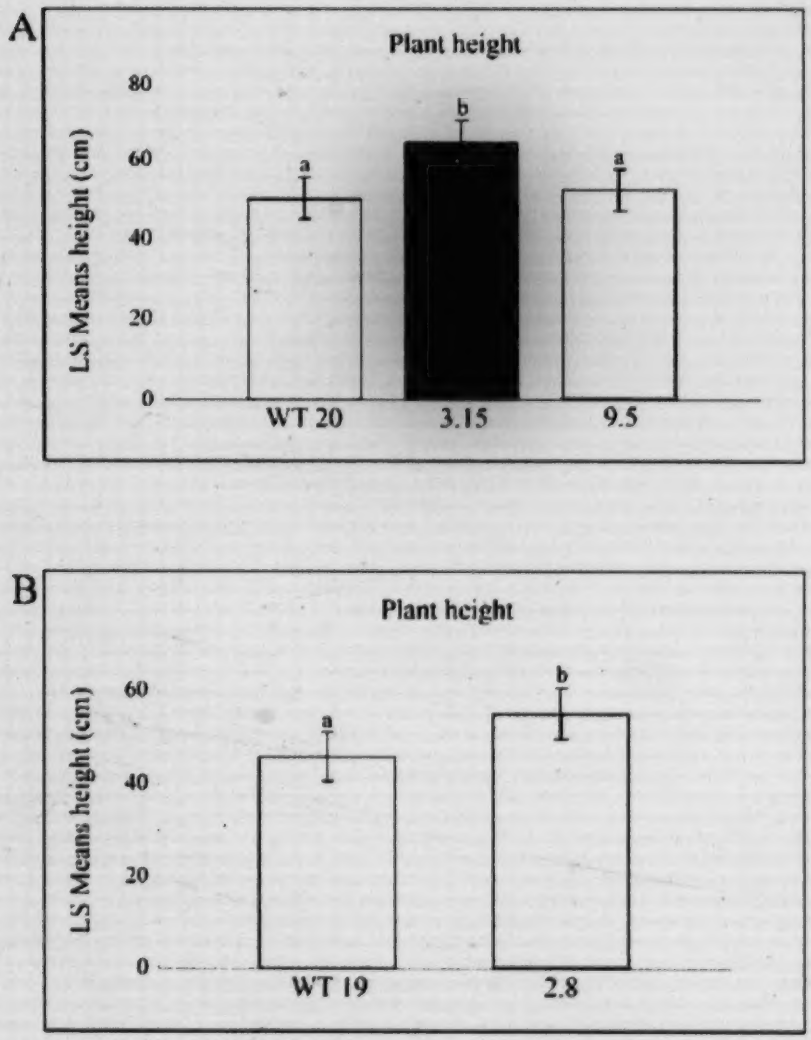

Figure 3.12 – Plant height of *ABR17* transgenic and WT *B. napus* adult plants 42 days after planting (DAP). Plant height of WT 20, 3.15 and 9.5 (A) and WT 19 and 2.8.2 (B). Three replicates were used with a total of 10 plants per replicate ($P < 0.05$).

Peace Region where the growing season is short, and in the southern part of the province where adequate precipitation is a problem. For example, if the plant switches from vegetative to reproductive growth too early seed production can be limited, because the plants do not have enough leaves and roots to provide energy to the developing flowers and seeds. On the other hand, if the switch to reproduction is delayed, even though the plant is large enough to gather photosynthates and nutrients, it may not have enough time to produce mature seeds. Numerous studies on the transition from reproductive growth to flowering have been undertaken (reviewed in Bernier and Perilleux 2005). Aside from environmental cues, like photoperiod and vernalization, CK and GA also affect the transition to flowering. Application of both phytohormones on the SAM of *Sinapis alba* activate the *SaMADS A* gene that may be involved in the transition to flowering (Bonhomme et al. 2000). For example, Chaudhury et al. (1993) found that *Arabidopsis ampl* mutant with high CK levels also demonstrated early flowering. Furthermore, Dewitte et al. (1999) reported that organ formation, including flowering is correlated with increased endogenous CK levels in tobacco. Indeed, we have demonstrated previously (Srivastava et al. 2006b) that transgenic *A. thaliana* plants expressing pea *ABR17* cDNA flower earlier and that this may be the result of enhanced, endogenous CK levels in that species (Srivastava et al. 2007). The earlier flowering observed in *ABR17*-transgenic lines investigated in this study may also be the result of enhanced CK levels although this suggestion must be verified through the determination of endogenous CKs in these lines. Regardless of the factors affecting this trait in the transgenic lines, the early flowering demonstrated by the transgenic lines is an attractive characteristic in agricultural species.

3.5 Relative expression of *ABR17* cDNA in transgenic lines

Even though RT-PCR experiments revealed the expression of the pea *ABR17* cDNA, the relative levels of expression in these lines could not be ascertained using that technique. In order to determine whether differences in the relative levels of expression between the two transgenic lines were contributing to the observed differences in germination as well as flowering, we performed qRT-PCR experiments. These experiments revealed that line 3.15 had the highest level of *ABR17* expression when compared to line 2.8, 2.8.2 and l9.5 (Figure 3.13). These results correlate well with the observed responses of these lines with respect to germination as well as earlier flowering. For example, the transgenic line 3.15 was observed to be the best when tested for germination in the presence of 275 mM NaCl, had the best developed roots and shoots (Figures 3.5A and 3.5B) and flowered earlier (Figures 3.10A and 3.11A) compared to line 9.5. The correlation observed between the levels of *ABR17* expression, which was approximately twice as high in line 3.15 (Figure 3.13), and the phenotypic characteristics of this line indicate that the observed phenomena are most probably due to the transgene expression. The differences between the transgenic lines with respect to the relative levels of expression may be the result of differences in the location of transgene integration i.e. positional effects.

3.6 Characterization of proteome–level differences between wild type and transgenic *B. napus*

In order to investigate the molecular mechanisms behind the observed *ABR17*-mediated effects in *B. napus* and to determine whether the introduction of the transgene led to undesirable changes in protein profiles, we performed 2-DE analysis of proteins extracted

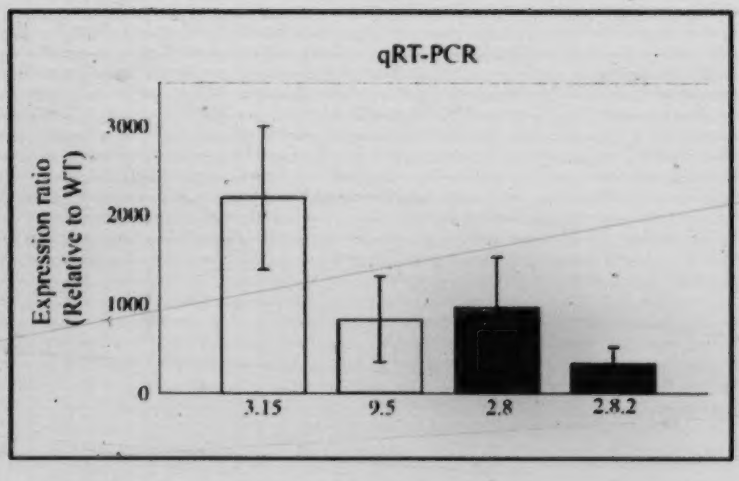

Figure 3.13 – Relative expression of *ABR17* in the transgenic lines.

from one transgenic line (3.15) and compared it to the proteome of WT 20. Specifically, we examined the proteome-level changes in the transgenic and WT lines at 2, 14, and 22 DAP. We selected transgenic line 3.15 for this comparison because it demonstrated the most transgene-induced changes (e.g. higher germination and earlier flowering) as well as having the highest level of *ABR17* expression. Representative gels from each of the three time points are shown in Figures 3.14-3.16. When comparing the transgenic line and the WT at 2 DAP the intensity of one protein spot was found to be significantly ($P < 0.05$) and reproducibly altered, while at 14 and 22 DAP three spots each were reproducibly and significantly altered. Among these 7 spots, all, with the exception of one, were down-regulated in the transgenic line. In addition, one spot was observed to be unique to the transgenic line at all time points. The identities of all 8 spots were established (Table 3.1) using tandem mass spectrometry and significant hits with scores above the threshold value were generated using mascot searches of the National Center for Biotechnology Information (NCBI) non-redundant database. A score above the threshold value indicates extensive homology or sequence identity ($P < 0.05$).

The unique protein spot observed in the transgenic line was identified as pea ABR17 (spot U; Table 3.1; Figures 3.14-3.16). Some of the other proteins identified in this study are involved in primary metabolism and photosynthesis. While, a number of them may also be involved in the stress response, including L-ascorbate peroxidase (APX), Kunitz-type cysteine protease inhibitor and glycine-rich RNA binding protein (GRP1) (Table 3.1). Furthermore, no proteins with any documented deleterious effects on humans or animals were identified by our 2-DE experiments.

Table 3.1- Details of proteins identified by electrospray ionization quadropole time-of-flight tandem mass spectrometry (ESI-Q-ToF MS/MS).

Spot No.	Protein identity	[a] PM %	[b] score	Sequence	[c] Access. No.	M_r/pI	Fold Change
1	Cruciferin CRU4 precursor (11S globulin) (12S Storage protein) [*Brassica napus*]	11%	294	R.CSGFAFER.F K.SNDNAQINTLAGR.T R.IEVWDHHAPQLR.C R.GLPLEVISNGYQISPQEAR.S	gi|461841	51630/ 7.70	-2.47±0.45
2	L-ascorbate peroxidase [*Raphanus sativus*]	19%	249	K.NYPAVSEEYQK.E K.NCAPIMVR.L R.TGGPFGTMR.F R.EDKPQPPPEGR.L R.EDKPQPPPEGR.L K.EGLLOLPSDK.A	gi|468733	27951/ 5.48	-2.92±0.82
3	CA1 (Carbonic Anhydrase 1) [*Arabidopsis thaliana*]	14%	182	K.YETNPALYGELAK.G K.YMVFACSDSR.V K.VISELGDSAFEDQCGR.C	gi|30678347	29827/ 5.54	+1.93±0.5
4	ROC4; peptidyl-prolyl cis-trans isomerase [*Arabidopsis thaliana*]	6%	150	K.FEDENFTLK.H R.TLESQETR.A R.TLESQSTR.A	gi|15228674	28532/ 8.83	-2.70±0.84
5	ATCTIMC (Cytosolic triose phosphate isomerase); [*Arabidopsis thaliana*]	6%	159	K.VASPAQAQEVHDELR.K K.VASPAQAQEVHDELRK.W K.VASPAQAQEVHDELRK.W	gi|15233272	27380/ 5.39	-1.91±0.30
6	Kunitz-type cysteine protease inhibitor [*Brassica oleracea* var. Botrytis]	5%	67	R.SNGGGLLPVPVK.L	gi|76150698	25069/ 5.83	-2.61±0.64
7	Glycine-rich RNA-binding protein GRP1A [*Sinapis alba*]	10%	122	R.GFGFVTFK.D R.SITVNEAQSR.G	gi|50900728	16063/ 5.21	-1.96±0.31
U	ABA-responsive protein [*Pisum sativum*]	15%	135	D.ADEIVP.K T.NYVLH.K G.DAALSDAV.R	gi|20631	20631/ 5.07	Unique

[a] Percentage identity between the amino acid sequences in the databases and those present in the MS/MS tag.

[b] Ion score is -10 log P, where P is the probability that the observed match is random. Individual ion scores > values indicate extensive homolog or identity (P <0.05).

[c] Mascot search result accession number using the National Center for Biotechnology Information (NCBI) and other databases.

All the proteins were identified at each time point and will be discussed in turn according to each developmental stage. At 2 DAP, spot 1 was identified as 12S globulin cruciferin (-2.47; Table 3.1; Figures 3.14A and 3.14B). This protein is the major storage protein of *B. napus* and accounts for between 50-60% of the total seed protein (Hoglund 1992).

At 14 DAP three spots were identified among which two (spots 2 and 4; Table 3.1) were observed to be reduced in intensity. Spot 2 was identified as APX (-2.92; Table 3.1; Figures 3.15A and 3.15B). APX is a primary antioxidant and an important scavenger of H_2O_2. Two molecules of ascorbate reduce H_2O_2 to water, generating two molecules of monodehydroascorbate (MDHA). In addition to scavenging H_2O_2, ascorbate is also involved in photoprotection, regulating photosynthesis, preserving activities of metal containing ions, and may also slow down DNA replication during oxidative stress to prevent DNA damage (reviewed in Noctor and Foyer 1998). Pnueli et al. (2003) reported that cytosolic ascorbate peroxidase *Apx1*-deficient *Arabidopsis* plants demonstrated an accumulation of H_2O_2 when grown under optimal conditions as well as, reduced growth, lower rate of photosynthetic activity, altered stomatal responses, and induction of heat shock proteins under light stress. The authors suggested that these responses might in fact mimic the effect that different environmental stresses have on plant growth and development through the presence of H_2O_2. The reduction in intensity of this spot in the transgenic line is perhaps contrary to what would be expected with respect to the observed salinity tolerance and may indicate that this enzyme is not responsible for the observed effects. It must be stressed that the proteome-level

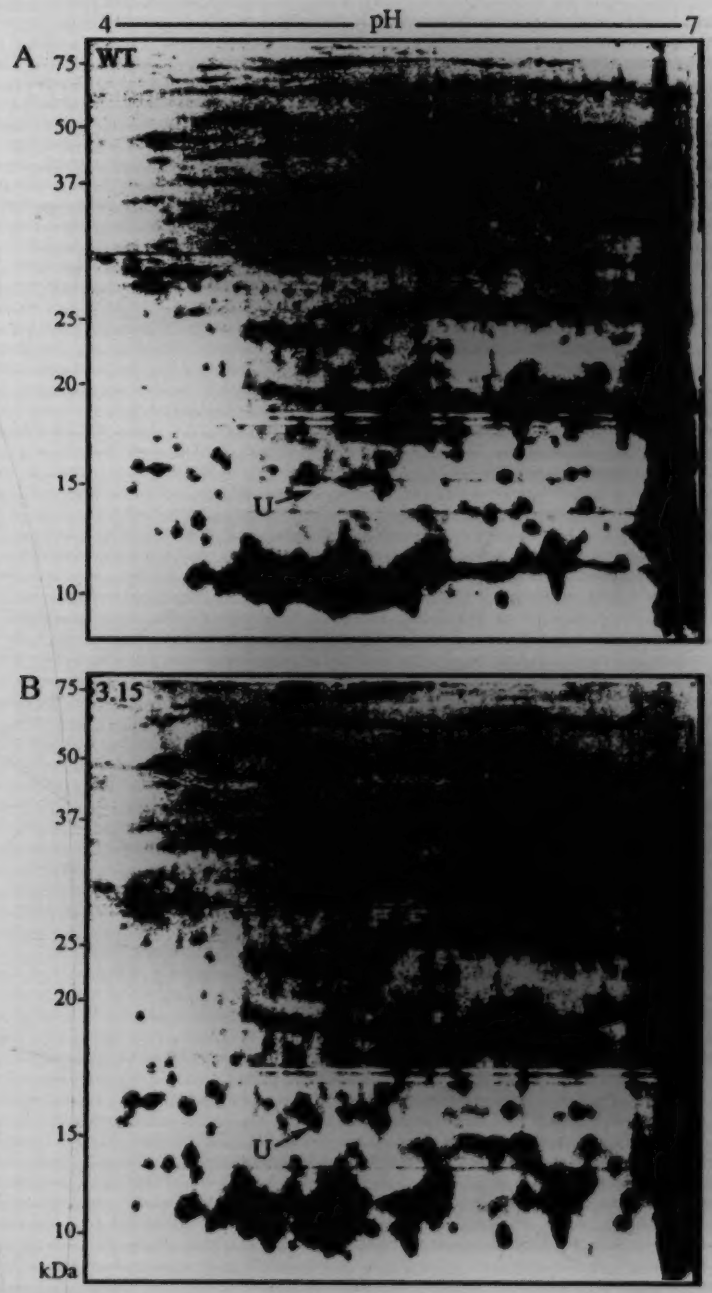

Figure 3.14 – Representative images of two-dimensional gels of WT and *ABR17* transgenic *B. napus* (3.15) protein extracts at 2 DAP. Expanded panels of spots selected for MS/MS identification are presented in Figure 3.17.

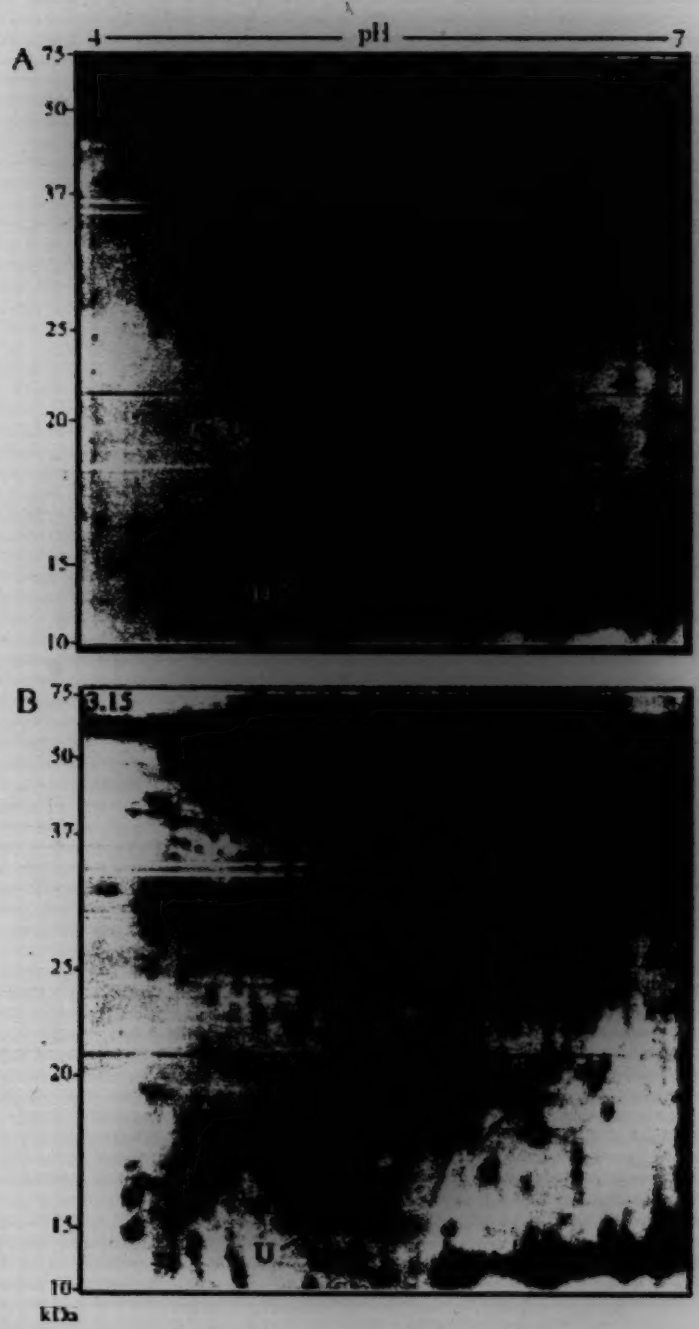

Figure 3.15– Representative images of two-dimensional gels of WT and *ABR17* transgenic *B. napus* (3.15) protein extracts at 14 DAP. Expanded panels of spots selected for MS/MS identification are presented in Figure 3.17.

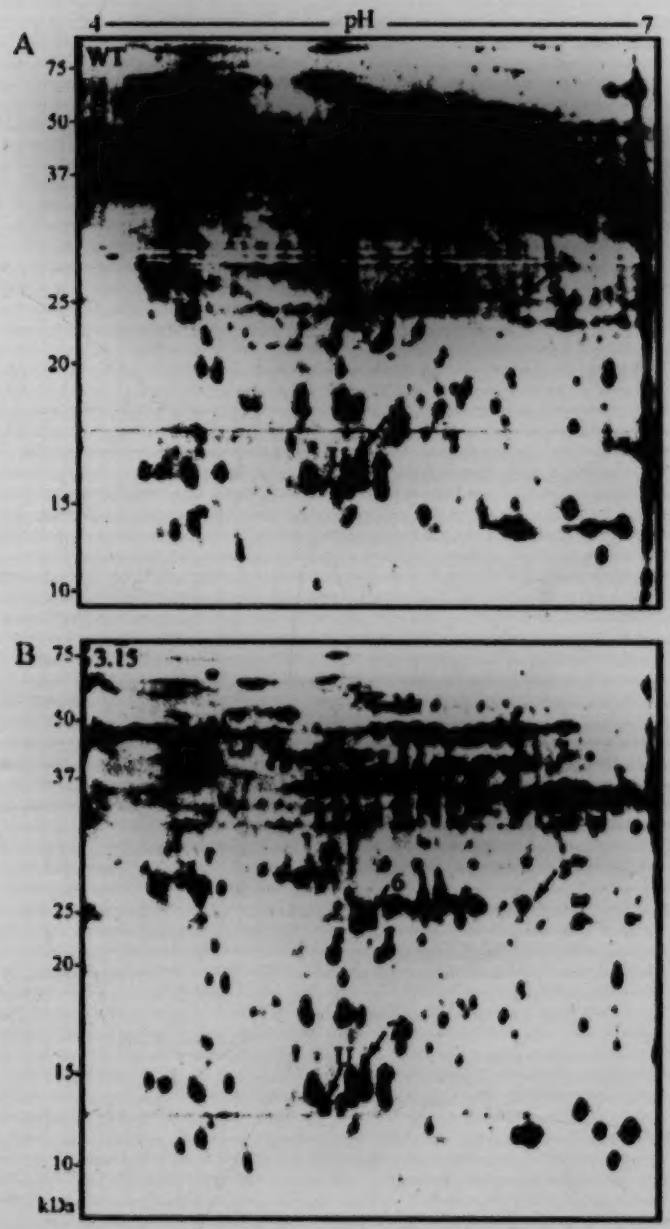

Figure 3.16– Representative images of two-dimensional gels of WT and *ABR17* transgenic *B. napus* (3.15) protein extracts at 22 DAP. Expanded panels of spots selected for MS/MS identification are presented in Figure 3.17.

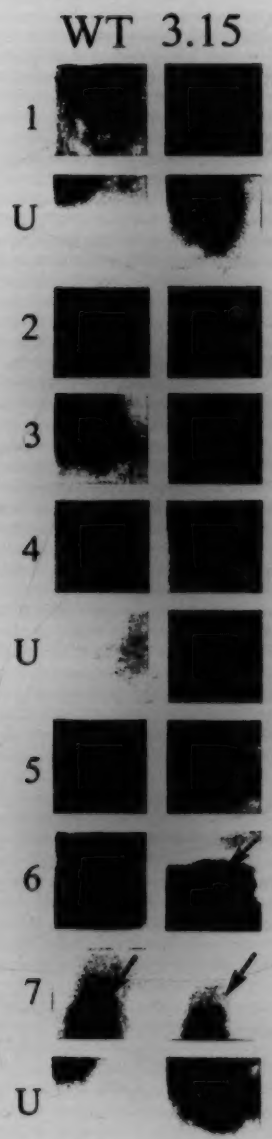

Figure 3.17 – Expanded panels of spots selected for MS/MS identification. One unique protein (ABR17) was identified in each developmental stage and is denoted as U in the expanded panels.

experiments were performed in the absence of stress and the results may be different in the presence of stress and must be investigated

Spot 3 was the only protein whose levels increased significantly ($P < 0.05$) in the transgenic lines and seems to correlate well with the observed phenotype. This spot was identified as carbonic anhydrase (CA), an enzyme that catalyses the reversible conversion of HCO_3^- to CO_2 (spot 3; +1.93; Table 3.1; Figures 3.15A and 3.15B). There are three families of CA, α-C, β-C and γ-C, and although all three enzymes are Zn^{+2} metalloenzymes and catalyze the same reactions, they have no sequence similarities (reviewed in Moroney et al. 2001). The highest amount of CA activity (β-C) is in the chloroplast stroma and it makes about 2% of the total leaf protein (reviewed in Moroney et al. 2001). The biosynthesis of CA is regulated in the plant by photon flux density (the number of photons striking a square meter surface every second), CO_2 concentration, and zinc availability (reviewed by Tiwari et al. 2005). For example, cytoplasmic and chloroplastic CA decreased when *Arabidopsis* plants were moved into darkness by 20 and 70 %, respectively (Fett and Colman 1994). Furthermore, β-C CA antisense *Arabidopsis* displayed no phenotype when grown in the soil, but when they were grown in MS plates all the antisense plants died. The authors speculated that under low CO_2 β-C is essential for the plant (unpublished results reviewed in Moroney et al. 2001). In addition, Yanyou et al. (2006) reported that plantlets with higher levels of CA have greater net photosynthesis and a faster growth rate. The rapid development of our *ABR17*-transgenic lines accompanied by a rapid rate of flowering could be the result of increased photosynthetic rate mediated by the enhanced levels of CA. A possible relationship between ABR17 and CA could be through the modulation of endogenous CK

levels by this protein which has been reported in transgenic *A. thaliana* expressing *ABR17* (Srivastava et al. 2007). In fact, the observation that exogenous CK application increased the expression of CA in root nodules of *Medicago* sp. (de la Pena et al. 1997) lends credibility to this suggestion. Future studies to measure photosynthetic rate and probe the amount of CA activity by enzyme assays and to establish whether or not these levels correlate with CK levels are warranted.

Spot number 4 was identified as peptidyl-peptidyl cis-trans isomerase (ROC4), from the cyclophilin enzyme family (-2.70; Table 3.1; Figures 3.15A and 3.15B). ROC4 is a chloroplast stroma cyclophilin strongly induced by light, while other rotamase cyclophilins (ROC 1-3, 5-6) are likely cytosolic and are expressed in numerous plant organs (Chou et al. 1997). Savitch et al. (2005) performed microarray analysis of *B. napus* overexpressing CBR/DREB1-like transcription factors and reported an increase in *ROC4* and *COR* gene mRNAs. These transgenic plants demonstrated increased cold tolerance and increased photosynthetic capacity. The reported involvement of both L-APX and ROC4 in the plant stress response suggests that these proteins may contribute to the observed abiotic stress tolerance in the transgenic lines, although their levels were lower in line 3.15. As a result, it would be prudent to investigate the levels of both APX and ROC4 enzymatic activity in WT and transgenic tissue under stress conditions.

At 22 DAP, three protein spots were identified from the shoot apex tissue. Spot number 5 was identified as cytosolic triose-phosphate isomerase (-1.91; Table 3.1; Figures 3.16A and 3.16B). This protein is involved in cytosolic glycolysis, catalyzing the reversible conversion of dihydroxyacetone phosphate to glyceraldehyde–3–phosphate. Spot number 6 was identified as Kuntiz-type cysteine protease inhibitor (-1.75; Table 3.1;

Figures 3.16A and 3.16B). These proteinase inhibitors are found throughout the plant kingdom, including *B. napus* (Ilami et al. 1997), *Arabidopsis* and cauliflower (Halls et al. 2006). Kunitz-type protease inhibitors have signal peptides indicating that they function within organelles or after being secreted from cells. In the case of both cauliflower and *Arabidopsis*, the inhibitor is part of a gene family that is expressed in response to environmental stress, such as drought (Halls et al. 2006). Similarly, in *B. napus*, BnD22 proteinase inhibitor was found to accumulate in leaves demonstrating drought tolerance (Ilami et al. 1997). Furthermore, these leaves showed less proteinase activity, a factor promoting leaf senescence. The increase in the proteinase inhibitor may be involved in decreasing protease activity and contributing to the delay in senescence under stress conditions (Ilami et al. 1997), suggesting that this protein may play a role in the observed stress tolerance in the *ABR17* transgenic lines, but it does not appear to play a role in the early flowering phenotype observed in the transgenic lines.

Spot number 7 was identified as glycine-rich RNA binding protein GRP1A (-1.96; Table 3.1; Figure 3.16A and 3.16B). GRP proteins are grouped into two major categories: one group contains a target sequence for the endoplasmic reticulum, while the other group contains an RNA binding sequence (RNP-1, RNP-2). The GRP1A protein shows homology to GRP proteins localized to the nucleus and has the two RNP-1 an RNP-2 sequences, indicating that it may have RNA-binding activity or be involved in RNA processing or control of gene expression. GRPs could also be involved in the plant stress response, because some GRP expression is responsive to ABA, salinity, wounding, cold and circadian rhythm (reviewed by Sachetto-Martins et al. 2000); it could be important in controlling RNA processing and/or RNA stability in response to

environmental changes (Shiozuka et al. 2006). The particular gene that was identified in our study shows homology to circadian controlled GRP1A from *Sinapis alba.* It was found to be localized in the nucleus in meristematic tissues, and is speculated to be involved in plant development. Our previous study on proteomic analysis of transgenic *ABR17 Arabidopsis* also revealed that glycine-rich RNA-binding protein in the transgenic line (Srivastava et al. 2006b), which once again suggests that this protein may also be involved in the stress response. Furthermore, its involvement in plant development, localization in meristematic tissue, and response to circadian rhythm indicate it may play some role in the advanced developmental phenotype we observed in the transgenic lines. Probing the relative expression levels of the GRP protein identified in our study may provide a mechanism for the early flowering we observed in the transgenic lines.

Although seven protein spots were reproducibly altered in the transgenic line, none of the identified proteins appear to pose any food safety/ human health concerns. Furthermore, the minimal proteome level changes demonstrated at all three time points may indicate substantial equivalence between the wild type and transgenic line at this level. Substantial equivalence indicates that the nutritional and toxic components of the two are lines are equivalent. However, the similarities at the proteome level do not replace the need for further nutritional, toxic and allergenic testing during the regulation of this and other transgenic crops. The minimal proteome level changes between the transgenic line and the wild type agree with Reufelt et al. (2006a; 2006b), who report that transformed *Arabidopsis* did not demonstrate any evidence of proteome level changes due to genetic modification exceeding that which was due to natural variation.

4.0 Conclusions

The results of this study demonstrate that the constitutive expression of *ABR17* cDNA in DH *B. napus* increases germination under both salinity and low temperature stress conditions, increases plant height and also promotes early flowering. Though the mechanism that *ABR17* imparts these changes is currently unknown, this study demonstrates the potential utility of *ABR17* in engineering these desirable traits in crop plants. Additional studies to establish the mechanism(s) behind the observed characteristics are warranted. Further examination of the identified proteins known to be involved in abiotic stress tolerance through qRT-PCR or enzyme assays in transgenic and WT plants under stress conditions may provide some answers. Examining the expression levels of CA in the transgenic and WT lines may provide a possible mechanism for the observations at the seedling and plant level. Measurement of photosynthetic rate in the transgenic and WT lines may also provide a clue to the role, if any, that CA performs. Our observation that ABR17 expression does not lead to dramatic changes in the *B. napus* proteome is an argument in favor of the substantial equivalence of our transgenic line with its WT counterpart at least at the level of the proteome. With current concern over the changes imparted to transgenic plants by genetic transformation, these findings are as important as the agronomic effects that ABR17 imparts. Plants with an ability to be seeded earlier, to germinate quicker, germinate in marginal soils and to flower faster will benefit the agricultural industry and positively impact both the producer and the consumer.

5.0 References

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